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ACETYLCHOLINESTERASE INHIBITORS ON THE SPINAL CORD

Subtitle: Actions of Organophosphates in the Mammalian Spinal Cord

FINAL REPORT

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This report describes studies on organophosphorus (OP) inhibitors of acetylcholinesterase (AChE) in the mammalian spinal cord in which the mechanism and site of action of the OPs on synaptic transmission were studied with selective agonists and antagonists of putative central neurotransmitters. Spinal cords isolated from neonatal rats 5- to 9-days old were hemisected and placed in experimental chambers. Electrodes attached to dorsal and ventral root pairs recorded the monosynaptic (MSR) and polysynaptic reflexes (PSR). The roles of N-methyl-D-aspartate (NMDA) and *non*-NMDA receptors in the generation of MSRs and PSRs and in the action of AChE inhibitors were examined. Utilizing specific receptor inhibitors it was found that the AChE inhibitors caused depression through muscarinic, not nicotinic receptors, that oximes antagonize AChE inhibitors by virtue of their anticholinergic actions, and not regeneration of ChE and that thyrotropin releasing hormone can effectively reverse the depression of reflex activity. The facilitation caused by the AChE inhibitors was found to be caused by a block of bicuculline-sensitive inhibition. In addition, it was found that NMDA and *non*-NMDA receptors are present at synapses between the dorsal root afferent neuron and the excitatory interneuron in the PSR and between the dorsal root afferent neuron and motoneuron of the MSR. Modulation of the NMDA receptor by Mg^{2+} alters the sensitivity of the spinal cord to AChE inhibitors: in the absence of NMDA antagonists, diispropylphosphorofluoridate and physostigmine are ineffective in depressing the MSR, but effectively depress the PSR. Reversal of both MSR and PSR depression by atropine suggests an interaction between muscarinic and glutamatergic receptors.

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FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised, 1978).

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SUMMARY

This report describes the actions of organophosphate (OP) and carbamate inhibitors of acetylcholinesterase (AChE) in the mammalian spinal cord, the reversal of those effects by known and putative antagonists (anticholinergics, oximes and peptides), actions on spinal inhibitory pathways and characterization of glutamatergic receptors in the isolated spinal cord. For these studies, spinal cords isolated from neonatal rats 5- to 9-days old were hemisected and placed in experimental chambers. Recordings were made from ventral roots under varying stimulation and recording paradigms to characterize the actions of these agents on excitatory and inhibitory pathways. Parallel studies on AChE were performed to determine the role of enzyme inhibition in the observed effects.

The characteristics of synaptic transmission were first established to determine optimal temperature (25°C), frequency of stimulation (generally 0.1 Hz) and analytical parameters. Dose-response curves for alteration of reflex transmission and of AChE by OPs and carbamates were obtained. Of all the inhibitors of AChE, only sarin (at low nM concentrations) facilitated the monosynaptic reflex (MSR). Some of the other AChE inhibitors also caused facilitation, but only in the presence of atropine. Mechanisms of facilitation could involve either blockade of spinal inhibitory pathways or enhancement of glutamate release from spinal afferent nerve terminals. We therefore examined the effect of sarin on the early, strychnine- and late, bicuculline-sensitive inhibitions in the spinal cord. Low concentrations of sarin (3-20 nM), which facilitated the MSR, reduced the late (bicuculline-sensitive) phase of inhibition but had no effect on the early (strychnine-sensitive) phase of inhibition. At concentrations of sarin ($\geq$ 30 nM) which depressed the MSR, the late phase of inhibition was either blocked to a lesser extent or enhanced. While the depression of the MSR has been attributed to activation of a muscarinic receptor and is unrelated to inhibition of AChE, the cause of the facilitation by sarin may be caused by a blockade of a bicuculline-sensitive inhibition which is most likely mediated by gamma-aminobutyric acid.

Antimuscarinic, but not antinicotinic agents, reversed the depression of the MSR caused by various OPs (and carbamates). Prevention of the depressant action of OPs by atropine occurred at nM concentrations while concentrations 100-200 times greater were required to reverse such actions. Thus, subtherapeutic concentrations of atropine are more effective in protecting against toxicity with OPs than in reversing their effects. Pirenzepine (an M_1 antimuscarinic agent), although less effective than atropine, reversed the depression of the MSR by sarin. Diazepam failed to reverse monosynaptic depression by diisopropylphosphorofluoridate (DFP), indicating that GABA receptors may not be involved in depression evoked by the AChE inhibitors. On the other hand, thyrotropin-releasing hormone (TRH; p-Glu-His-Pro-amide), a putative neuromodulator in the ventral horn of the spinal cord, completely reversed the depression of the MSR caused by DFP and by sarin suggesting that peptidergic or glutamatergic transmission is vital in reversing OP-induced depression of spinal synaptic transmission.

The ability of pralidoxime, trimedoxime (TMB-4), and diethyxime to reverse sarin-induced depression of the MSR was examined. These oximes reversed the depression in a concentration-dependent manner. The reversal was not accompanied by a regeneration of AChE activity in the spinal cord suggesting that the reversal of sarin-induced depression of synaptic transmission in the spinal cord by these oximes was unrelated to an effect on AChE or the accumulation of

acetylcholine. It is more likely that the reversal resulted from blockade of cholinergic (muscarinic) receptors in the spinal cord.

To further understand the depressant action of the OPs in the spinal cord, it was first necessary to characterize polysynaptic transmission in the isolated spinal cord, a study not previously reported. Mg^{2+}, a known antagonist of the *N*-methyl-D-aspartate (NMDA) receptor-channel, was withdrawn from the physiological solution, resulting in a dose-dependent increase in MSR magnitude followed by a second potential, the polysynaptic reflex (PSR). Whereas the MSR was relatively resistant to NMDA antagonists [2-amino-5-phosphonovalerate (APV) and 2-amino-7-phosphonoheptanoate (AP7)], the PSR was markedly reduced in a dose-dependent manner by APV, AP7 and by 6,7-dinitro-quinoxaline-2,3-dione (DNQX). The magnitude of the MSR maximally decreased only 20-30% at concentrations of Mg^{2+} (1.3 mM), APV (10 μM) and AP7 (10 μM) which completely depressed the PSR. These results suggest that the MSR is subserved mostly (70%) by non-NMDA (kainate and quisqualate) receptors and to a small extent (30%) by NMDA receptors, the extent of which is governed by the local Mg^{2+} concentration. The PSR appears to be wholly subserved by NMDA receptors. These studies also indicate that both NMDA and *non*-NMDA receptors are present at the synapses of both the afferent neuron and the excitatory interneuron in the PSR, and the afferent neuron and the motoneuron in the MSR. DNQX is a 15- to 20-fold more potent antagonist of *non*-NMDA than NMDA responses.

The modulation of the NMDA receptor by Mg^{2+} significantly affects the ability of OPs and carbamates to depress monosynaptic transmission. In the absence of Mg^{2+}, DFP and physostigmine, but not sarin, were ineffective in depressing the MSR while significantly depressing the PSR. The results may suggest that the glutamatergic receptors exist in various forms and that one can modulate their predominance in any one pathway. The latter findings, together with the paradoxical observation that increasing the concentration of AChE inhibitor in the absence of Mg^{2+} eventually results in reversal of depression, are not easily explained at this time and must await further investigation.

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I. STATEMENT OF PROBLEM UNDER STUDY

The overall goal of this research has been to determine the mechanism by which various inhibitors of acetylcholinesterase (AChE) affect synaptic transmission in the mammalian spinal cord and to establish modes by which these effects can be prevented and/or reversed. The study has focused on the ability of these agents to potentiate and/or depress mono- and polysynaptic transmission in the neonatal rat spinal cord. To pursue this course of study, the actions of organophosphorus (OP) and carbamate inhibitors of AChE on the isolated cord were first characterized on the spinal monosynaptic reflex (MSR) with full dose-response curves. Similarities between the OP inhibitors of AChE and the carbamates were determined through observations of spinal monosynaptic transmission. The ability of sarin to alter pre- and/or postsynaptic inhibition in the cord as a mechanism for facilitation was pursued. Various mechanisms which might explain the observed depression of spinal monosynaptic transmission by the OPs were examined with the use of anticholinergics (*viz.*, muscarinic and nicotinic antagonists), diazepam, thyrotropin-releasing hormone, carbamates (physostigmine and pyridostigmine), and oximes. The ability of AChE regenerators (*i.e.*, oximes) to restore monosynaptic transmission in cords exposed to OPs was examined and did not correlate with AChE activity. The protective effect of carbamates on the lethal action of the OPs and their ability to hasten recovery from OP toxicity was assessed in the spinal cord. A study on the ability of thyrotropin-releasing hormone to reverse OP-induced toxicity shed light on the role of peptidergic and glutamatergic transmission in OP-induced toxicity. Finally, studies were performed to clarify the action of OPs on *N*-methyl-D-aspartate (NMDA) and *non*-NMDA-mediated spinal activity.

II. RATIONALE

The mechanism and site of action of the AChE inhibitors were studied in spinal cords maintained *in vitro* in a nearly physiological state. This preparation is most suited to the studies because the external milieu is easily controlled with respect to O_2-CO_2 levels, electrolyte and drug concentrations, and because stable recordings can be made for extended periods ($>$ 10 hr) without interference from respiratory movements or vascular pulsations engendered in spinal preparations *in vivo*. Unlike central neurons studied in tissue culture, those in isolated cord preparations maintain synaptic connections with other segmental and suprasegmental neurons (excitatory and inhibitory) such that the influence of various catecholaminergic, cholinergic, peptidergic, and glutamatergic neurons on the actions of the AChE inhibitors can be assessed.

III. INTRODUCTION

The neurotoxic action of anti-acetylcholinesterase (AChE) compounds has received much attention because of the potential use of organophosphorus (OP) compounds as chemical warfare agents and the extensive use of carbamates as pesticides. The *irreversible*[§] inhibitors of AChE comprise a group of OP compounds which includes sarin (isopropyl methylphosphonofluoridate), soman (pinacolyl methylphosphonofluoridate), tabun (ethyl N-dimethylphosphoramidocyanidate), diisopropylphosphorofluoridate and VX (*O*-ethyl *S*-[2-(diisopropylamino)ethylmethylphosphonothioate). The carbamates, on the other hand, are typified by pyridostigmine (PYR) and physostigmine (PHY) (quaternary and tertiary carbamates, respectively) which *reversibly* inhibit AChE and have qualitatively similar pharmacological actions. Although OP agents such as echothiophate and diisopropylphosphorofluoridate (DFP) have previously found limited medical application in the pharmacotherapy of glaucoma and strabismus, it is the carbamates which are more widely known for their therapeutic applications. The latter agents have been used in the treatment of cholinergic syndrome, myasthenia gravis, and memory loss and as adjuncts during the reversal of neuromuscular block following surgery where they act to prolong the duration of ACh in the synapse.[133]

Many investigators have sought to explain OP action based on enhanced or depressed cholinergic activity subsequent to inhibition of AChE. Among the reasons for this assertion is that past knowledge about the mechanism(s) of action of OP compounds was confined to and derived from studies on *cholinergic* function in animal tissues, and clinical observations of persons accidentally or purposefully exposed to OPs. The subsequent overstimulation of cholinergic function at both central and peripheral sites then resulted in either facilitation or depression of function. The intoxication resulting from the action of both *reversible* carbamate and *irreversible* OP inhibitors of AChE therefore has been generally believed to result from the inhibition of AChE. These agents are in fact known to act at muscarinic (autonomic) and nicotinic (somatic) synapses in the peripheral nervous system where the magnitude of synaptic responses is either enhanced or reduced as an apparent function of the degree to which ACh accumulates.[133] But, the OPs and some carbamates also have effects at cholinergic receptors in the central nervous system where their action is associated with desynchronization of the electroencephalogram, hypokinesia and catalepsy, tremors and convulsions.[85]

The possibility then arose that the *reversible* inhibitors of AChE might protect from OP intoxication. A number of studies in animals have shown, in fact, that the carbamates are effective in preventing intoxication by irreversible OP inhibitors of AChE apparently through protective carbamylation of AChE.[11,30,40,62,66,68,70,73,128] The reversible blockade of AChE prevents the more toxic irreversible OP compounds from phosphorylating or phosphonylating the enzyme, affording sufficient time for the OP compound to undergo uncatalyzed hydrolysis. When atropine is used in conjunction with the carbamates to prevent muscarinic crisis, the protective efficacy of the carbamates is increased. The carbamates, however, have effects at the neuromuscular junction which are unrelated to inhibition of AChE and the resultant accumulation of ACh. Those direct effects are mediated through the ion channel of the nicotinic receptor

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[§] The term "*irreversible*" refers to the inexorable inhibition of AChE once "ageing" of the enzyme occurs.

where PYR acts as a weak agonist and PHY blocks the channel in its open conformation.[1] Studies such as this suggest that there may be sites other than AChE and the cholinergic receptor at which the OPs and carbamates act to manifest their toxic effects, particularly in the central nervous system.

The studies on the spinal action of AChE inhibitors began in the late 1930s and it quickly became apparent that there were conflicting results with the carbamate-type compounds. The observed effect on synaptic transmission was dependent upon the particular compound, its concentration and the duration of exposure, some producing both acute toxicity and chronic neuropathies. For example, low doses of PHY consistently enhanced the patellar reflex in both normal and atropinized cats without affecting the polysynaptic reflex (PSR) while 'prostigmine' (*neostigmine*) and various semisynthetic analogs of PHY always depressed the reflex.[123-125,140] Ten-fold higher doses of PHY were reported to depress the MSR, an effect which was attributed to the depolarization of primary afferents rather than to stimulation of spinal inhibitory neurons.[55] Although the excitant effects of low dose PHY and depressant effects of the other carbamates were atropine-*insensitive*, the depressant effect of high dose PHY was mecamylamine-*sensitive*. Like PHY, intrathecal injections of DFP in cats facilitated the patellar reflex at low doses but depressed the reflex at 10-fold higher doses.[20] Although the latter actions were attributed to inhibition of AChE, no enzyme measurements were performed. It is significant however that the convulsions caused by DFP and tetraethyl pyrophosphate in animals were diminished by atropine.[42] Furthermore, the persistence of significant amounts of AChE in the spinal cord of animals during blockade of reflex activity implied that a mechanism other than inhibition of AChE was responsible for the depression seen with DFP and tetraethyl pyrophosphate.[119] Tabun, another OP compound, had differential effects on reflex activity: it depressed the MSR but facilitated polysynaptic activity in cats.[77] DFP, on the other hand, did not affect the MSR. Based on these and other studies, the possibility was raised that the OP compounds might have pharmacological actions other than their common ability to inhibit AChE.[77,78] Indeed, 20 years after that initial suggestion, it was reported that DFP directly blocked the ion channel of the nicotinic ACh receptor, an action unrelated to the inhibition of AChE.[91,92]

But support for the role of AChE in the toxic actions of AChE inhibitors has also stemmed from the efficacy of certain quaternary bispyridinium oximes which were developed to interact chemically with the esteratic site of AChE. The efficacy of these oximes in treating OP toxicity has been attributed to their ability to reactivate AChE.[60,74,75,144] These compounds were therefore suggested as adjuncts along with a cholinolytic drug in counteracting OP toxicity.[74,75] However, the ability of the quaternary oximes to reactivate brain AChE as a mechanism for antidotal action in OP intoxication is controversial. On the one hand, quaternary reactivators such as pyridine-2-aldoxime methiodide (pralidoxime; 2-PAM) exhibit low lipophilicity and do not easily penetrate the blood-brain barrier, although one study has shown results to the contrary.[59] Even pro-PAM, the lipophilic precursor of pralidoxime, only provided marginally greater survival against DFP than did 2-PAM and no protection against sarin[21] or paraoxon in mice, even though it regenerated brain AChE.[72,127] Unlike most oximes, diethyxime {S-[2-(diethylamino)ethyl]4-bromobenzothiohydroximate hydrochloride} is a tertiary reactivator of AChE and a reportedly universal antidote against OP intoxication at both central and peripheral sites.[90] The availability of a lipophilic oxime has therefore aroused some interest in one's ability to antagonize the central effects of OP compounds. Moreover, the structural similarity between the oximes,

nicotine and the nicotinic conformation of ACh[129] suggests that the antidotal action of the oximes might be attributed to a direct antagonism of ACh at nicotinic receptors as well as reactivation of phosphorylated AChE. Additional support for this assertion are findings that oximes possess antagonist activities at both nicotinic and muscarinic receptors[3,93,129]. In fact, both pralidoxime and HI-6, another AChE reactivator, can modify the functional properties of the ion channel of the nicotinic ACh receptor.[2]

Although the probable site of OP- and carbamate-induced depression of segmental transmission has been examined, the mechanism of depression and facilitation has hitherto eluded us. Obvious possibilities include a direct effect on the motoneuron (*e.g.*, depolarization), the release of a putative excitatory transmitter (*e.g.*, glutamate), and blockade of inhibition. One possible site that has not yet been implicated in the spinal action of OPs is the glutamatergic receptor which is intimately involved in excitatory synaptic transmission in the spinal cord. It is generally, although not universally, accepted that at least three subtypes of glutamatergic receptors exist, each named for their preferred agonist: quisqualate, kainate, and NMDA. Although the availability of selective agonists and antagonists for the NMDA receptor have allowed its characterization, our understanding of neurotransmission mediated through *non*-NMDA (quisqualate and kainate) receptors has been hampered. In the mammalian spinal cord, the use of selective receptor agonists and antagonists has established that the spinal segmental PSR is mediated, at least in part, by NMDA receptors.[26,34,150] Similar experiments on the MSR using *non*-NMDA receptor agonists and nonselective glutamate antagonists have suggested that the MSR is mediated by quisqualate and/or kainate receptors.[5,26,34,35,63,83,99,117] Monosynaptically activated segmental motoneurons appear to have both NMDA and *non*-NMDA receptors in the developing rat spinal cord.[150] The possibility existed, therefore, that the OPs might depress spinal synaptic transmission either directly or indirectly through NMDA and *non*-NMDA receptors and might cause facilitation through blockade of inhibition.

Previous work from this laboratory has demonstrated noncholinesterase actions of OPs which have excitant and/or depressant effects on the spinal cord. Sarin, for example, has a biphasic action on the spinal cord of neonatal rats, *in vitro*, facilitating the MSR at low concentrations (< 50 nM) and enhancing frequency-dependent depression but depressing the MSR at higher concentrations.[145] Soman, on the other hand, only depressed the MSR in the cat and in the isolated cord preparation of the neonatal rat.[144,146] Inhibition of AChE was apparently not involved in the action of OPs since prior inhibition of AChE by DFP failed to alter sarin's ability to facilitate or depress segmental transmission.[145] Similar results were reported in the superior cervical ganglia of rats where prior irreversible inhibition of AChE by DFP did not alter the concentration-dependent depression of synaptic transmission seen with soman.[148]

Since the OPs appear to exert different actions, we were first interested in examining the effect of DFP in the isolated spinal cord of the neonatal rat, correlating these actions with AChE activity and examining the ability of cholinergic antagonists to alter OP-induced responses in the spinal cord. Subsequently, the actions of sarin and, to varying extents, soman, tabun and VX and of two carbamate inhibitors of AChE (*i.e*, PHY and PYR) were examined on reflex activity in the spinal cord. The ability of several cholinergic antagonists, oxime regenerators of AChE, thyrotropin-releasing hormone (TRH) and of NMDA and *non*-NMDA antagonists to interact with the two classes of AChE inhibitors was examined as was the interaction between the OPs and

carbamates on spinal reflex activity. As part of this work, spinal synaptic activity in the cord was also characterized. Thus, optimal temperature and frequency of stimulation were examined, pre- and postsynaptic inhibition was characterized and the role of NMDA and *non*-NMDA receptors in the mono- and polysynaptic pathway was assessed. This report summarizes the investigations carried out over a three and one-half year period that were supported by this U.S. Army Medical Research and Development Command contract (DAMD17-86-C-6030).

IV. METHODS AND MATERIALS

The housing of the animals and preparation of the isolated spinal cord from neonatal rats was similar to that already described.[37,115,116,135] Briefly, 6- to 9-day-old rat pups of either sex (obtained from dams of the Wistar strain; Charles River Breeding Laboratories, Inc., Kingston, NY) or pregnant dams of the Wistar strain (Charles River Breeding Laboratories, Kingston, NY) were obtained at 12-16 days gestation, housed individually in polycarbonate cages with Betachips as the bedding and allowed food (Purina Chow) and water *ad libitum*. The room was environmentally controlled with a 12-hr light/dark cycle at 22-23 °C and constant humidity. The rat pups were allowed to remain with the dam from the day of birth (day 0) until the day of the experiment (day 6-9). The pups were anesthetized with diethyl ether and their spinal columns were removed and placed in a petri dish containing oxygenated physiological solution. The spinal cord from the midthoracic to the midsacral level was removed along with the corresponding dorsal and ventral roots, hemisected in the longitudinal plane, and the dura was removed. The longitudinally hemisected cord was then transferred to a small Plexiglas bath (total volume, approximately 1 ml) through which oxygenated physiological solution maintained at 25 $\pm$ 0.5 °C was constantly perfused at 2-3 ml/min. Cords from either male or female rat pups were used in most experiments except those which involved thyrotropin-releasing hormone (TRH) in which only male rat pups were used.[37]

A. Stimulation and Recording

A L_{3-5} dorsal root was stimulated supramaximally (duration, 0.2 msec; frequency, 0.1 Hz) through a suction electrode attached to a stimulus isolation unit (Digitimer, Model DS-2) which was controlled by a Digitimer (Medical Systems, Model D4030). The reflex response evoked in the corresponding ventral root was amplified (WP Instruments, New Haven, CT, Model M707; Axoclamp-2, Axon Instruments, Burlingame, CA), displayed on an oscilloscope (Tektronix, Beaverton, OR), acquired by an on-line computer (IBM-AT) utilizing pCLAMP (Version 4.2; Axon Instruments, Burlingame, CA), and stored on floppy diskettes for off-line analysis. Alternatively, the potentials were recorded on an FM tape recorder (Racal, Model 4 store DS, Sarasota, FL) or on a video cassette recorder (SONY, Model SL-HF900) via a digital pulse code modulator (SONY, Model PCM501ES) for off-line analysis. To obtain stable D.C. potentials, the bath was grounded via an indifferent electrode. The preparation was allowed to stabilize in the chamber for 2 hr to allow recovery from the dissection and also to provide a steady-state response. The reflex evoked in this manner is a stable waveform and similar in latency and time course to that seen *in situ*. Once the suction electrodes sealed to the roots ($\approx$ 1-2 hr), little decrement (< 10%) was seen in the reflex over a 10-hr period at the optimum temperature of 25 °C and with adequate oxygenation.

Control reflex activity elicited at 0.1 Hz was recorded from a ventral root prior to exposing the cord to each concentration of drug. The cords were superfused with different concentrations of agent and then washed with physiological solution until the control amplitude was attained, or given either fixed or varied concentrations of antagonist together with the agent under examination. In other sets of experiments the cord was pretreated with different concentrations of antagonist for 20 to 30 min and then simultaneously exposed to varying concentrations of agent under consideration. Appropriate control experiments with vehicle (propylene glycol for DFP) or with the various antagonist drugs were performed to assess any potential actions by themselves. Each observation was obtained by measuring the area and/or latency of the traces produced by signal-averaging 5-10 successive reflexes. Latency was defined as the time interval between the onset of stimulation and the first detectable point on the upstroke of the reflex. All recordings were made only after this steady-state response had been achieved. The dose-response data (semicumulative) for various drugs were obtained after 30-min exposures. Each observation for a given concentration was from a different cord; up to six concentrations were applied to individual cords at progressively higher concentrations without washing between applications.

For the *inhibition of reflex transmission*, two adjacent dorsal roots and a ventral root between the L_3 and L_5 segments were attached to suction electrodes. Supramaximal stimulation of a dorsal root elicited a response in the corresponding ventral root after a latency of 3-5 msec. The control amplitude of the MSR was first determined from an average of 3-5 reflexes elicited at 0.1 Hz prior to instituting any conditioning protocol. At this frequency of stimulation, there was neither potentiation nor depression of the reflex. Conditioning stimuli of supramaximal strength were delivered to an adjacent dorsal root at intervals of 1, 3, 5, 7, 10, 15, 20, 30, 50, and 70 msec) before the test stimulus was applied to elicit the MSR in the ventral root.[39] The conditioning stimulus by itself did not evoke any potential in the ventral root. A test MSR without a conditioning stimulus was also obtained just before and again after a MSR associated with a conditioning stimulus to confirm the stability of the MSR throughout the experiment. The experimental protocol thus involved recording 3-5 control responses followed by 10 or more pairs of conditioning-test (C-T) responses, each of which was preceded and followed by a single unconditioned MSR.

B. Measurement of Acetylcholinesterase Activity

Spinal cords without their roots were isolated from 8- to 10-day-old rat pups of either sex, weighed, and homogenized in ice-cold 0.05-M phosphate buffer at pH 7.4 using a microhomogenizer (Micro-Metric Instrument Company, Tampa, FL). Control AChE activity was measured by Ellman's method[52] at 25 $\pm$ 1 °C, using an ultraviolet-visible spectrophotometer (LKB-4050, Ultrospec II, Princeton, NJ). In a parallel set of experiments, the effect of the AChE inhibitors, oximes (10 μM) alone, and in combination with an OP (after sarin pretreatment) on AChE activity was determined. In the experiments with sarin, the spinal cords were exposed to the OP for 2 hr, after which the cords were superfused with each oxime (10 μM) + OP for an additional 30 min. The residual AChE activity was then determined as above. In some cases, cords were superfused with physiological solution for 2 hr after exposure to OP whereupon AChE activity was determined.

C. Solutions and Drugs

The physiological solution contained (mM): NaCl, 124; KCl, 5.0; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $NaHCO_3$, 15.0; and glucose, 11.0. $MgSO_4$ (0-1.3 mM) was added as necessary without compensating for the small change in osmolarity. The temperature was maintained at 25 $\pm$ 0.5 °C. The solution was bubbled with 95% O_2-5% CO_2 and had a pH of 7.3 irrespective of the Mg^{2+} concentration. The spinal cords in which only the MSR was to be recorded were prepared and maintained in a physiological solution containing Mg^{2+} and then subjected to each experimental paradigm. In those experiments in which the polysynaptic reflex (PSR) was also recorded, the spinal cords were prepared and maintained in a physiological solution free of Mg^{2+} and then subjected to each experimental paradigm.

A stock solution of 1 M DFP (Sigma Chemical Co., St. Louis, MO) was prepared in propylene glycol (Sigma). Thereafter, all subsequent dilutions were made in physiological solution. Sarin, soman, tabun (2 mg/ml in physiological saline), and VX (1 mg/ml in physiological saline) were obtained from the U.S. Army Medical Research and Development Command and kept frozen (-80 °C). Stock solutions of the potent OPs (10^{-4} M) were prepared in physiological saline at the time of the experiment. DL-APV, DL-AP7, atropine sulfate, benactyzine, pirenzepine, pralidoxime, trimedoxime, physostigmine and thyrotropin-releasing hormone (Sigma), diazepam and pyridostigmine (Hoffmann-LaRoche) and diethyxime (gift of Dr. Das Gupta) were prepared as stock solutions (10^{-2} M) in distilled water. DNQX was obtained from Tocris Neuramin (Essex, United Kingdom) and dizocilpine (MK-801) was a gift from Merck Sharp & Dohme Research Laboratories (Harlow, Essex, United Kingdom). Stock solutions of DNQX were prepared in alkalinized distilled water. All stock solutions were stored frozen and were thawed just prior to use when they were diluted as required in physiological solution. For the enzymatic assay of ChE, acetylthiocholine iodide and 5,5'-dithiobis-(2-nitrobenzoic acid) (Sigma) were prepared in 0.05 M phosphate buffer at pH 7.4, refrigerated, and used within the week.

D. Analysis

In early experiments, taped responses were amplified, digitized and stored on removable hard disks and then analyzed using a MINC\DECLAB 23 computer (Digital Equipment Corp., Maynard, MA). In later experiments, acquisition and analysis of signals were performed using an IBM computer in which the amplified signals were digitized, averaged, stored, and analyzed using pCLAMP (Axon Instruments, Burlingame, CA). Reflex activity was quantitated by measuring the area under the reflex curve since it has generally been assumed to more accurately reflect the total population of neuronal activity evoked in the spinal cord.[65] In some experiments the recovery of the MSR area (A) from OP-induced depression was calculated according to the formula: A_o-A_i/A_c-A_i x 100 where A_o = Area after treatment with an oxime, A_i = Area after inhibition, and A_c = Area of the control (*i.e.*, 100%). Data are presented as the mean $\pm$ SEM and were statistically analyzed using repeated measures analysis of variance (one-way ANOVA) followed by an appropriate test such as Dunnet's test or Newman-Keuls test. A comparison of the data only between two groups was performed with paired or unpaired Student's *t* test. *P* values of 0.05 or less were considered statistically significant. Details of the computer-assisted analysis appear elsewhere.[135]

The concentration of drugs which produced a 50% inhibition (IC_{50}) of the MSR or PSR under a given set of conditions (*e.g.*, 1.3 mM Mg^{2+}) is the mean of four to five determinations from different cords. Each determination was interpolated from a first-order linear regression line, using the linear portion of the dose-response curve. In most cases the dose-response curves were constructed in a semicumulative manner (*vide supra*). For example, a control reflex was recorded at a given [Mg^{2+}], 1 μM DNQX was then applied for 30 min, and the reflex was recorded. DNQX was then rinsed from the bath with a drug-free solution of the same [Mg^{2+}]. The cord was then superfused with a solution containing a higher [Mg^{2+}] for 20 min before repeating the exposure to DNQX for up to three different [Mg^{2+}]. All data are expressed as the mean $\pm$ S.E.M. where N = number of observations. Details of the computer-assisted analysis can be found elsewhere.[135]

V. RESULTS

A. Diisopropylphosphorofluoridate on the Isolated Spinal Cord

1. *Effect on the Monosynaptic Reflex*

The primary effect of DFP was a concentration-dependent reversible depression of the MSR. At 0.01-0.1 μM, DFP had no effect on the MSR, but when the spinal cord was superfused with 1 μM DFP, the MSR was depressed to 92% of control (Fig. 1). At 100 μM DFP, the MSR was 47% of control, and raising the concentration to 1 mM resulted in complete blockade. Propylene glycol, the vehicle for DFP, had no effect on the MSR at a concentration (0.1% w/v) equal to that found at 1 mM DFP. The concentration of DFP which produced 50% depression (IC_{50}) of the MSR was estimated from the curve relating DFP concentration to area of the MSR (Fig. 1) and found to be about 80 μM. This depression could be reversed by vigorous washing over a 2-hr period (Fig. 2). Recovery from DFP-induced depression was dependent upon the concentration of DFP: the higher the concentration, the longer the washing period before recovery (Fig. 2). For example, at 100 μM DFP, the depression could be reversed in 2.5 hr while at 500 μM there was only a partial recovery after 180 min.

2. *Reversal and Antagonism of Monosynaptic Depression*

The depression caused by DFP could be completely and quickly (< 10 min) antagonized by exposure to atropine or benactyzine (Figs. 3 and 4). For example, 100 μM DFP depressed the MSR to about 40% of control (Fig. 4). Simultaneous exposure to either atropine (0.5 μM) or benactyzine (0.5 μM) completely restored the MSR to normal. Initial studies with (+) tubocurarine and mecamylamine revealed that they had no effect by themselves on the reflex. Whether they alter OP-induced depression will be examined in an additional set of experiments.

The depression caused by DFP could be reduced or prevented by pretreatment for 20 min with low concentrations of atropine (Fig. 5). At 30 nM, for example, atropine provided complete protection against the depressant effect of all concentrations of DFP tested from 10 to 500 μM. Intermediate effects were obtained at 10 nM atropine (Fig. 5) while 2 nM atropine had no effect on the response to DFP (data not shown). At 5 nM atropine (data not shown), only the

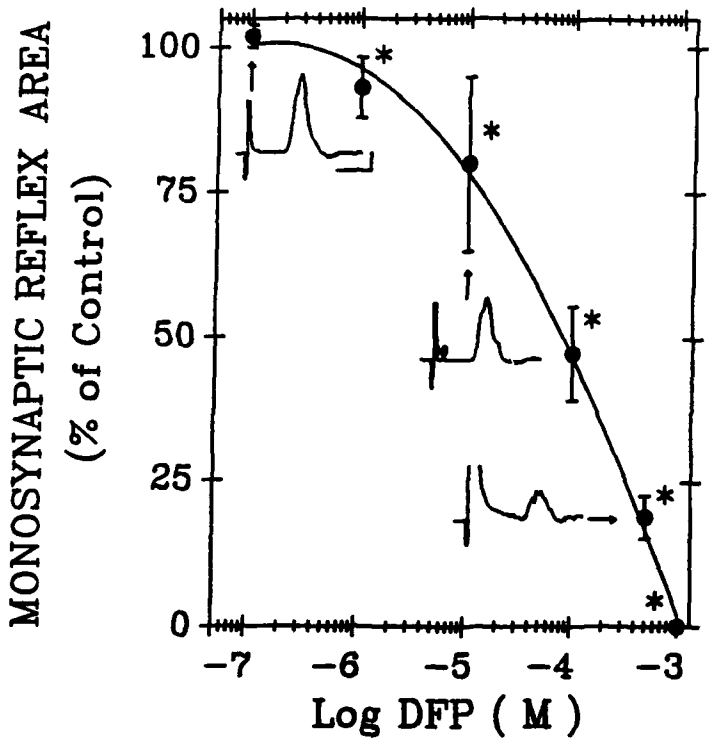

Fig. 1. Depression of the MSR by DFP. The spinal cord was first exposed to DFP (100 μM) for 15 min and then to DFP. The traces shown are representative of the responses obtained in the spinal cord of the neonatal rat *in vitro*.

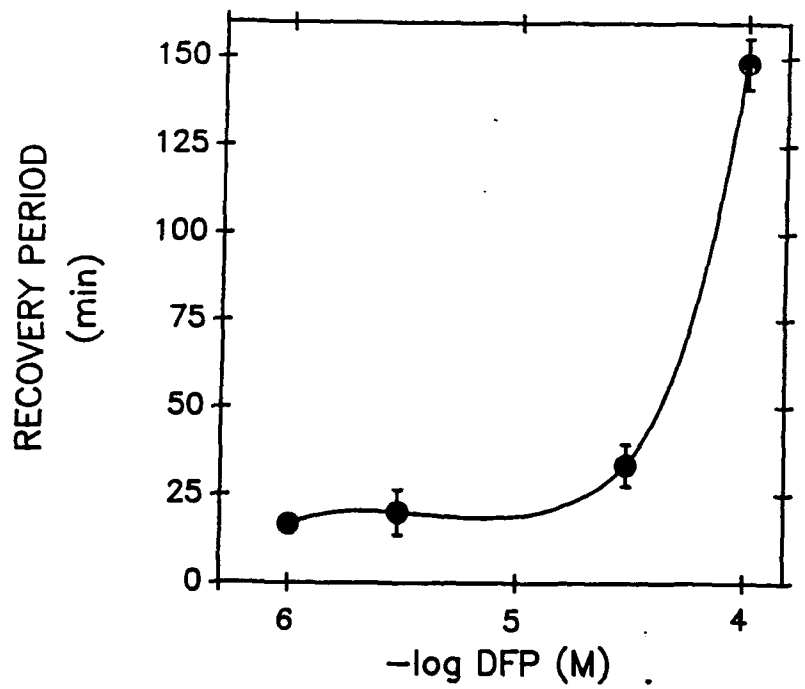

Fig. 2. Time course of recovery of the MSR after exposure to DFP. The cords were first exposed to DFP at various concentrations for 15 min and then washed with drug-free physiological solution at a rate of 3 ml/min. The values presented are the means ± SE in three preparations at each concentration.

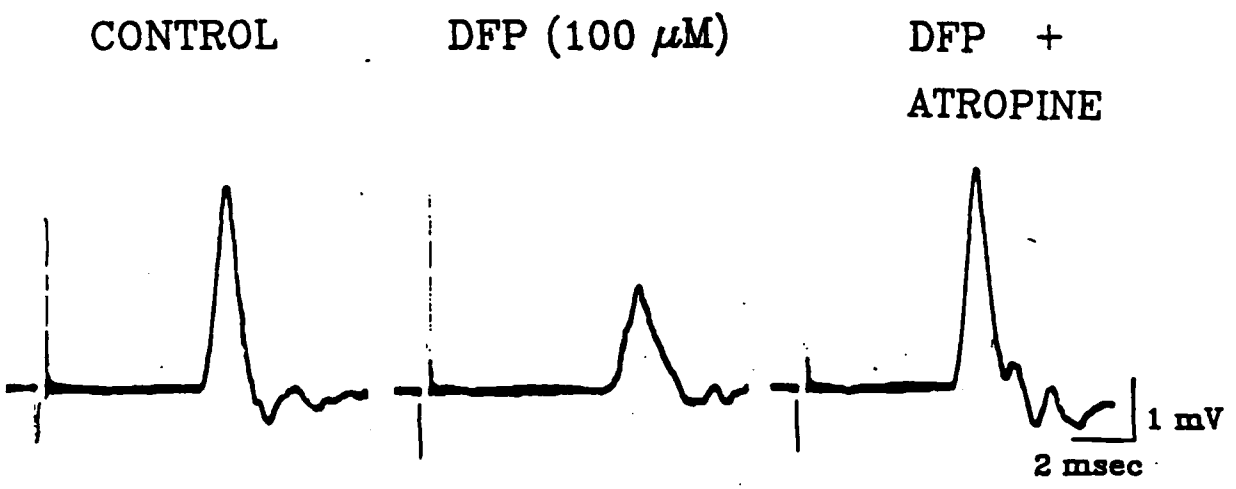

Fig. 3. Reversal of DFP-induced depression of the MSR by atropine. The cord was first exposed to DFP (100 μM) for 15 min and then to DFP + atropine (0.5 μM) for 10 min. The traces shown are representative of the responses obtained in the spinal cord of the neonatal rat *in vitro*.

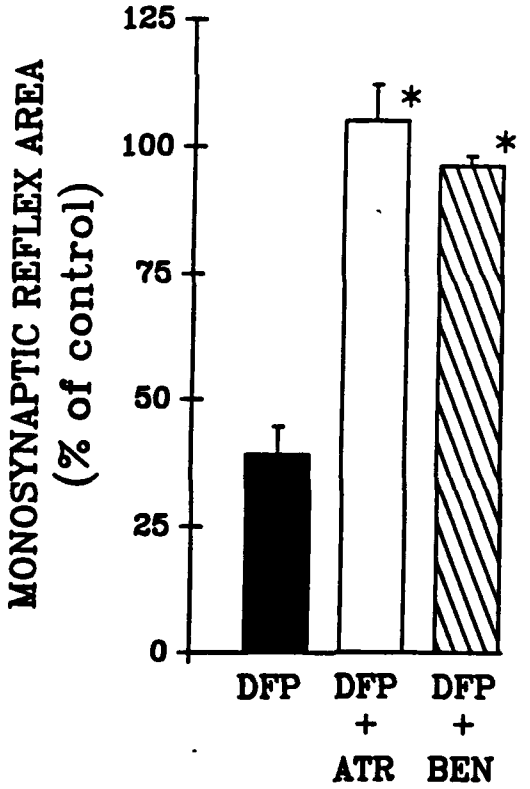

Fig. 4. Effect of anticholinergic agents on the depression of the MSR by DFP. The values shown represent the means ± SE of 3-6 spinal cords. In each case, a spinal cord was superfused with DFP (100 μM) for 15 min after which it was exposed to DFP + atropine (ATR; 0.5 μM), DFP + benactyzine (BEN; 0.5 μM) for an additional 15 min. The asterisks indicate statistical significance ($P < 0.001$) when compared to DFP alone (Student's t test).

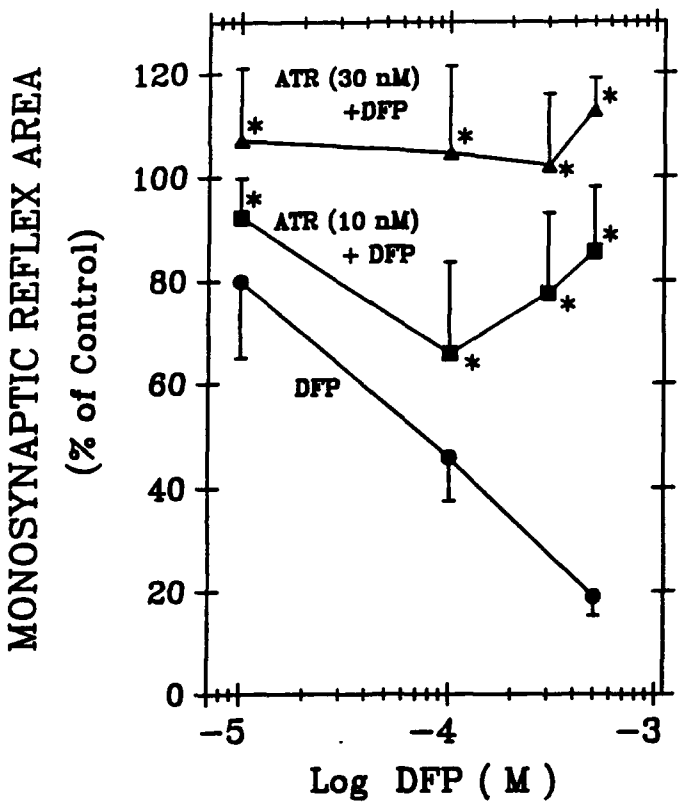

Fig. 5. Pretreatment with atropine reduces DFP-induced depression of the MSR. The spinal cords of 6- to 9-day-old neonatal rats were first superfused either with DFP alone (15 min) or with 10 or 30 nM atropine (ATR) for 20 min. The cords that were exposed to atropine were then superfused with atropine + DFP for an additional 15 min at each concentration of DFP. Each point is a mean ± SE from 3-4 cords. The asterisks indicate statistical significance ($P < 0.001$) of atropine pretreatment when compared to DFP alone (ANOVA).

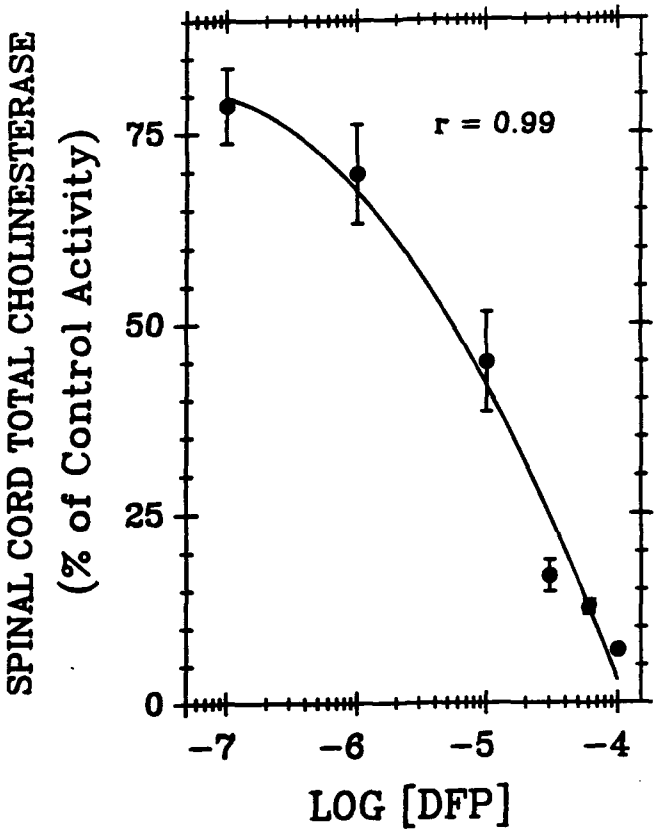

Fig. 6. Concentration-dependent depression of total spinal cholinesterase activity by DFP. Individual spinal cords were exposed to different concentrations of DFP for 15 min. Estimations of cholinesterase activity were then performed on the homogenate from these cords obtained from 8- to 9-day-old rats. The values shown are the means ± SE of 3-4 experiments at each concentration of DFP.

responses at 300 and 500 μM DFP were significantly different ($P < 0.01$) from the DFP control. The curves for pretreatment with atropine on DFP-induced depression were not, however, shifted in a parallel fashion when compared to the DFP control curve. For example, at 10 nM atropine, the values at 10 and 100 μM DFP were shifted in a parallel fashion but at 300 and 500 μM DFP there was less depression. The shape of the DFP curves in the presence of 5-30 nM atropine are themselves parallel with each other but not with the DFP control curve.

3. *Effect on Cholinesterase Activity*

ChE activity was unaffected at 0.1 μM DFP, but when the cord was treated with 100 μM DFP for 15 min, there was nearly complete inhibition of the enzyme (Fig. 6). The estimated IC_{50} for DFP was 7.0 μM. The inhibition of ChE by 100 μM DFP persisted even after washing the cords for more than 120 min.

B. Sarin on Spinal Excitation and Depression

1. *Effect of Sarin on the Monosynaptic Reflex*

Sarin caused a concentration-dependent facilitation and depression of the MSR. At 1 pM to 2 nM, sarin had no effect on the MSR, but when the spinal cord was superfused with 5 to 20 nM sarin, there was a concentration-dependent facilitation of the MSR (Figs. 7 and 8). At 50 nM and above, sarin depressed but did not completely block the MSR. The maximal facilitation was about 50% at 20 nM sarin, while maximal depression to about 25% of control occurred at 200 nM and persisted even when the concentration was raised to 1 μM. The concentration of sarin which caused 50% inhibition of the reflex was estimated to be about 90 nM. After continued exposure to sarin at concentrations as high as 1 μM, the preparations were simultaneously exposed to sarin (1 μM) + atropine (500 nM) (Figs. 7 and 8). This resulted not only in the reversal of sarin-induced depression but in a significant ($P < 0.05$) potentiation which reached values nearly 20% above control, as if atropine had unmasked the facilitatory action of sarin. Subsequent washing resulted in a small but insignificant reduction in the magnitude of the MSR 1 hour later.

2. *Reversal of Sarin-Induced Spinal Effects by Cholinergic Antagonists*

Atropine reversed sarin-induced depression in a concentration-dependent manner but did not affect facilitation caused by sarin. When cords were exposed to sarin (200 nM) for 60 min, the MSR was maximally depressed to 25% of control (Fig. 9). Thereafter, the cord was simultaneously exposed to various concentrations of atropine added to the bathing solution which contained sarin. The concentration of atropine was increased every 30 min beginning at 2 nM and reaching 500 nM. The ability of atropine to reverse sarin-induced depression became apparent at 5 nM, with half-maximal reversal occurring at about 50 nM (+ 200 nM sarin). The reflex returned to the control value at 500 nM atropine (Fig. 9). It would be interesting to determine the efficacy of benactyzine in reversing or preventing sarin-induced monosynaptic depression.

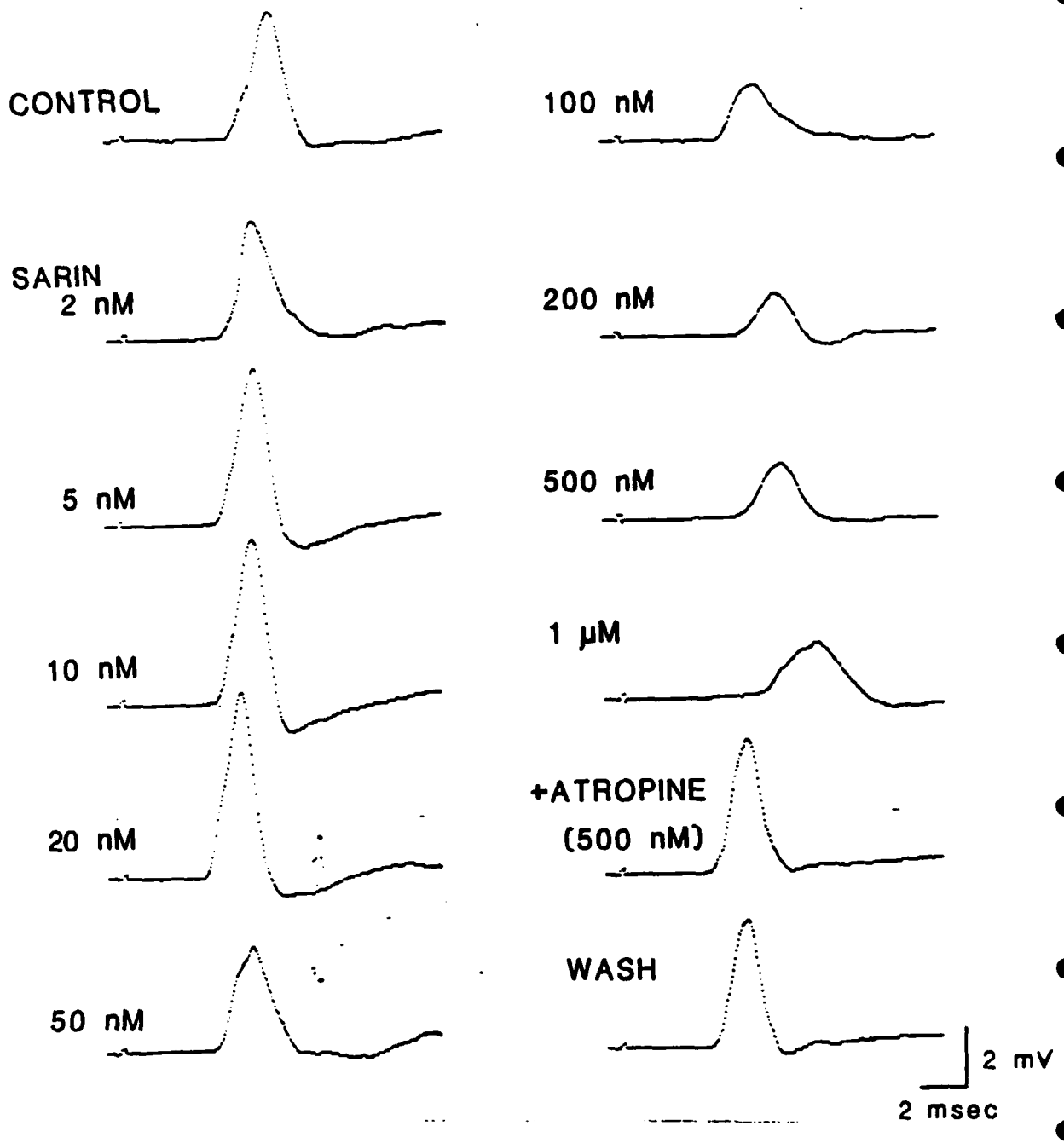

Fig. 7. Biphasic effect of sarin on the MSR. The recordings were made before and 30 min after exposure to each concentration of sarin. Each panel is the digitized, signal-averaged response of 10 traces evoked at 0.1 Hz. After exposure to 1 μM sarin, the cord was exposed to atropine (500 nM) + sarin (1 μM) for 30 min and then washed for 30 min with physiological solution.

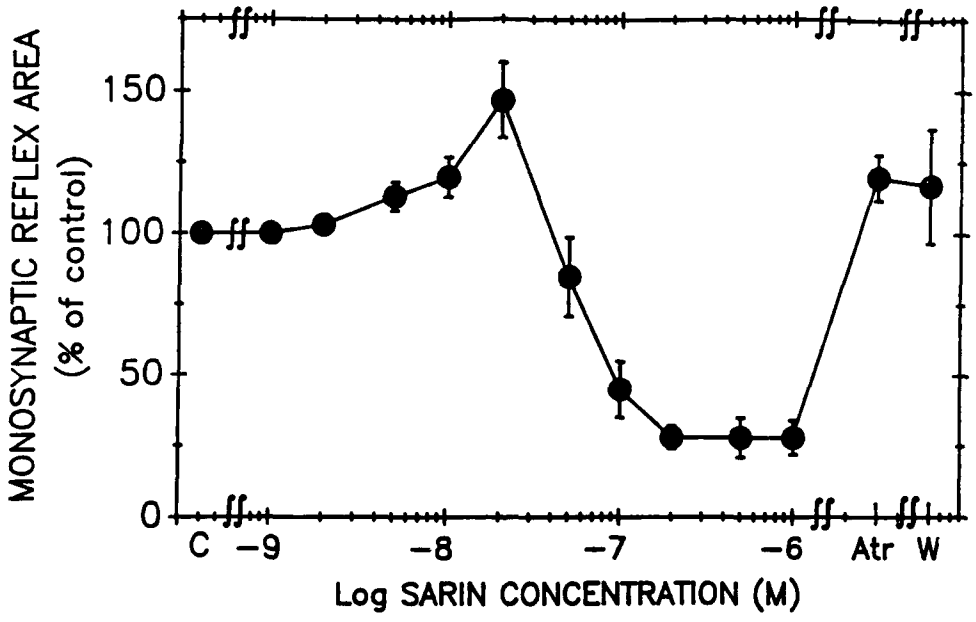

Fig. 8. Cumulative dose-response curve for the effect of sarin on the MSR. After the control period (C) the cords were exposed to increasing concentrations of sarin every 30 min. The values presented are the mean ± S.E.M. of 3-5 experiments where the mean value at each concentration of sarin was determined from the integrated area of the signal-averaged response of 10 potentials. The values shown between 5 and 1000 nM sarin are all significantly different ($P < 0.05$; ANOVA) from control as is the value for atropine + sarin.

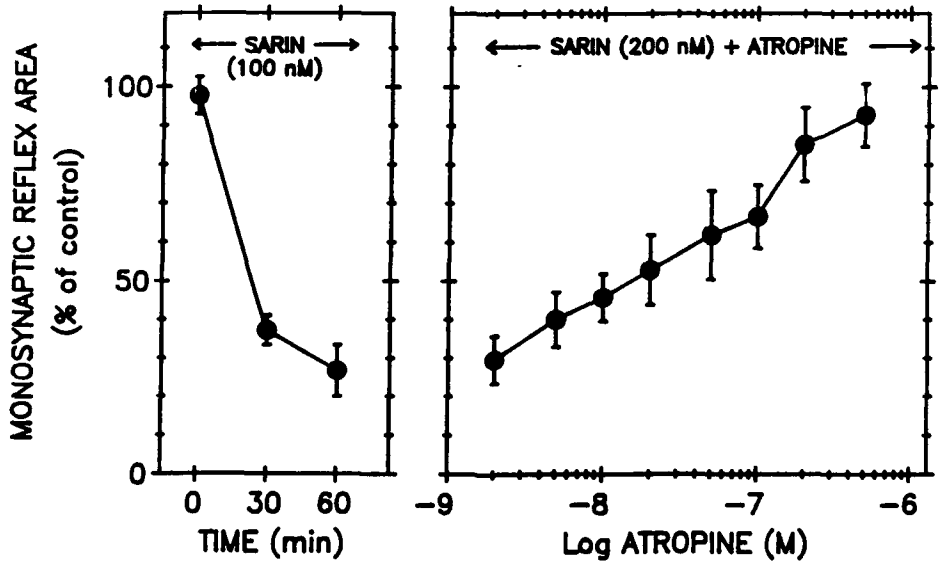

Fig. 9. Reversal of sarin-induced depression of the MSR by atropine. The cords were first exposed to sarin (200 nM) for 60 min (left panel) and then to sarin (200 nM) + atropine beginning at 2 nM (right panel). Recordings were made before and 30 min after every change in the concentration of atropine. The data presented are mean values of 10 signal-averaged responses from each preparation.

Pirenzepine (100 nM), on the other hand, never restored the MSR to more than 62% of control, regardless of whether the concentration of pirenzepine was further increased (Fig. 10). The addition of atropine (500 nM) to the solution containing both sarin (100 nM) and pirenzepine (100 nM) resulted not only in the restoration of the reflex to control but in the facilitation of the response to 35% greater than control. Interestingly, neither atropine, pirenzepine (M_1-specific) nor benactyzine had any effect on the magnitude of the MSR when applied at concentrations from 10 nM to 10 μM.

3. *Prevention of Sarin-Induced Depression by Anticholinergic Drugs*

The depressant action of sarin could be prevented when the spinal cord was pretreated with concentrations of atropine which were ineffective in completely reversing the effect of sarin. At 10 nM atropine, the depressant action of sarin at concentrations ranging from 100 nM to 500 nM was almost completely blocked and never exceeded 10% (Fig. 11). Pretreatment with atropine was therefore 50 times more effective in preventing sarin-induced depression than in reversing the depression once it had been established.

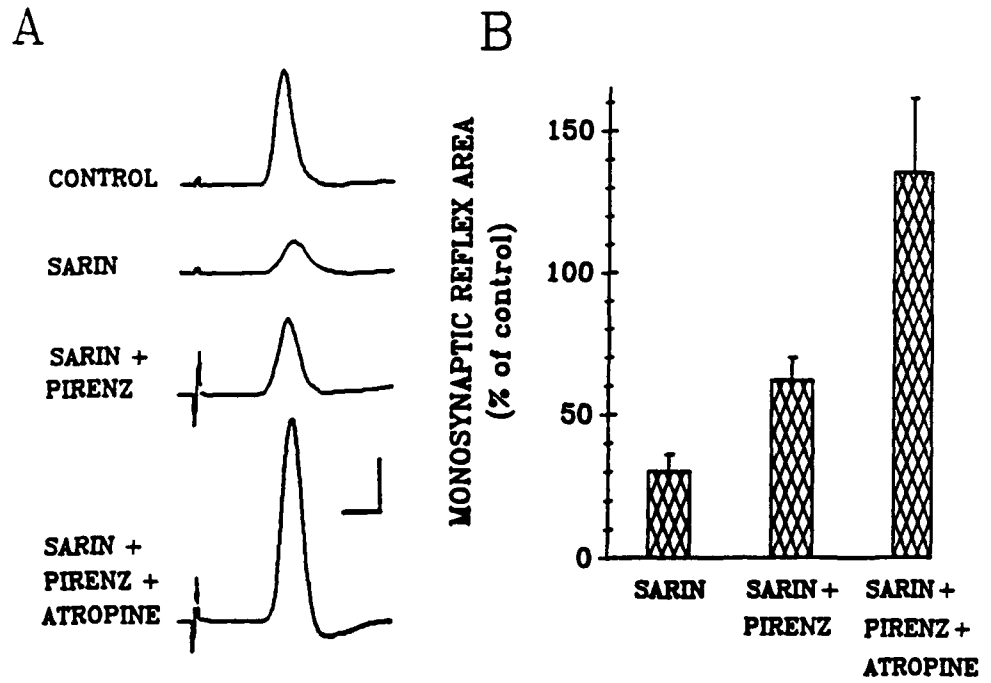

Fig. 10. Reversal of sarin-induced depression of the MSR by pirenzepine. Spinal cords were first exposed to sarin (100 nM) for 60-75 min and then successively exposed to sarin + pirenzepine (200 nM) for 15 min, followed by sarin + pirenzepine (100 nM) + atropine (500 nM) for an additional 10-15 min. The cords were subsequently washed with normal physiological solution. A typical experiment is shown in (A) were the depression by sarin was maximal at 75 min. Exposure to 100 nM pirenzepine resulted in only partial reversal of the sarin-induced depression while the response was facilitated when atropine was added to the solution containing sarin + pirenzepine. The data in (B) summarizes the results of four such experiments where the values shown are the mean ± S.E.M.

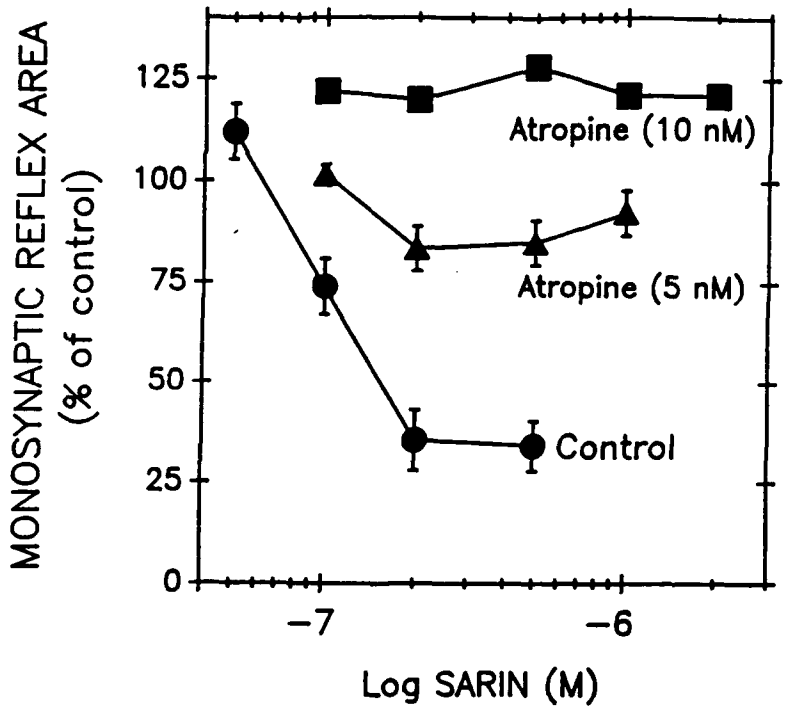

Fig. 11. Blockade of sarin-induced depression of the MSR by atropine. The cords were exposed either to sarin alone for 30 min (control) or pretreated with atropine for 30 min and then exposed to sarin + atropine for an additional 30 min. Each point is the mean of 3-5 experiments at each concentration of atropine or of the sarin control. The curves in the presence of atropine were significantly different ($P < 0.05$) from those of sarin alone.

Although antimuscarinic drugs proved effective in reversing the depression of the MSR by sarin, antinicotinic drugs did not. Mecamylamine, a tertiary antinicotinic agent, had no effect on the MSR at concentrations from 100 nM to 10 μM. When cords were pretreated with 100 nM mecamylamine for 30 min and then to mecamylamine + sarin (at concentrations from 100-500 nM), the depression caused by 100 nM sarin was not evident, but higher concentrations of sarin (200-500 nM) significantly depressed the MSR in a manner similar to that of sarin alone (Table 1; compare with Fig. 8). In additional experiments, (+)-tubocurarine (10 nM to 10 μM) had no effect on the MSR and did not reverse the depression caused by maximally depressant concentrations of sarin (data not shown). In addition, neither atropine nor other anticholinergic drugs affected facilitation caused by low concentrations of sarin.

4. *Effect of Sarin on Frequency-Dependent (Homosynaptic) Depression*

In the absence of drugs, reflex transmission in spinal cords from the neonatal rat was depressed during trains of stimuli delivered at frequencies greater than 0.1 Hz. The depression was most pronounced at frequencies greater than 0.5 Hz (Fig. 12). Sarin reduced frequency-dependent depression of the MSR at 20 nM (a facilitating concentration) and 200 nM (a depressant concentration), whether or not atropine was present. The magnitude of the MSR during exposure to sarin was, however, influenced by the prior excitatory history of the spinal cord. Thus, in a quiescent preparation, the effect of a depressant concentration of sarin was less than that in a preparation which had been active recently.

TABLE 1

Effect of mecamylamine pretreatment on sarin-induced depression of the monosynaptic reflex in neonatal rats

The spinal cords were exposed to mecamylamine alone for 30 min and then to mecamylamine + sarin for an additional 15 min. The results with mecamylamine + sarin were compared with the effects of mecamylamine alone and with those of sarin alone for similar time periods.

Mecamylamine (nM)	Sarin (nM)	Monosynaptic Reflex Area (% of Control)[a]
100	0	100.4 ± 2.4[b]
100	100	96.3 ± 7.2[b,c]
100	200	34.6 ± 3.0[d]
100	300	25.4 ± 3.2[d]
100	500	22.6 ± 5.2[d]

[a] The values presented are the mean ± S.E.M.; n = 3).
[b] $P > 0.05$ with respect to pre-drug and mecamylamine controls (Student's t test).
[c] $P < 0.05$ with respect to sarin alone (Student's t test).
[d] $P > 0.05$ with respect to sarin alone (Student's t test).

5. *Alteration of Cholinesterase Activity by Sarin*

To substantiate the notion that the effect of sarin and of other OP agents on the spinal cord were not due to inhibition of AChE, we examined the effect of DFP on reflex transmission, on AChE activity and on sarin-induced depression in the spinal cord. DFP (0.1 mM) depressed the MSR by 50% and prolonged washing partially reversed the depression (Fig. 13) as did the addition of atropine. At the same time, this concentration of DFP irreversibly inhibited AChE activity in the cord for at least 4 hr after washing DFP from the bath. The subsequent exposure of the DFP-treated spinal cords to sarin (2 nM to 1 μM) resulted in the same degree of facilitation and depression seen in spinal cords not previously treated with DFP (Fig. 13); compare with Fig. 7).

6. *Clonidine on Depression of the Monosynaptic Reflex by Sarin*

The activation of central α-adrenergic receptors on cholinergic nerve terminals by clonidine has proven effective against soman in mice.[16] We sought to determine whether this agent might also be effective in preventing the effects of sarin in the spinal cord. Clonidine (0.1-10 μM) by itself had no effect on the MSR when applied for up to 30 min. Sarin (100 nM) depressed the MSR (area) to 48.6 ± 5.0 % of control (n = 10). When the cords were exposed to 100 nM sarin + 10 μM, the MSR decreased to 34.2 ± 6.3 % of control ($P < 0.05$ with respect to sarin alone).

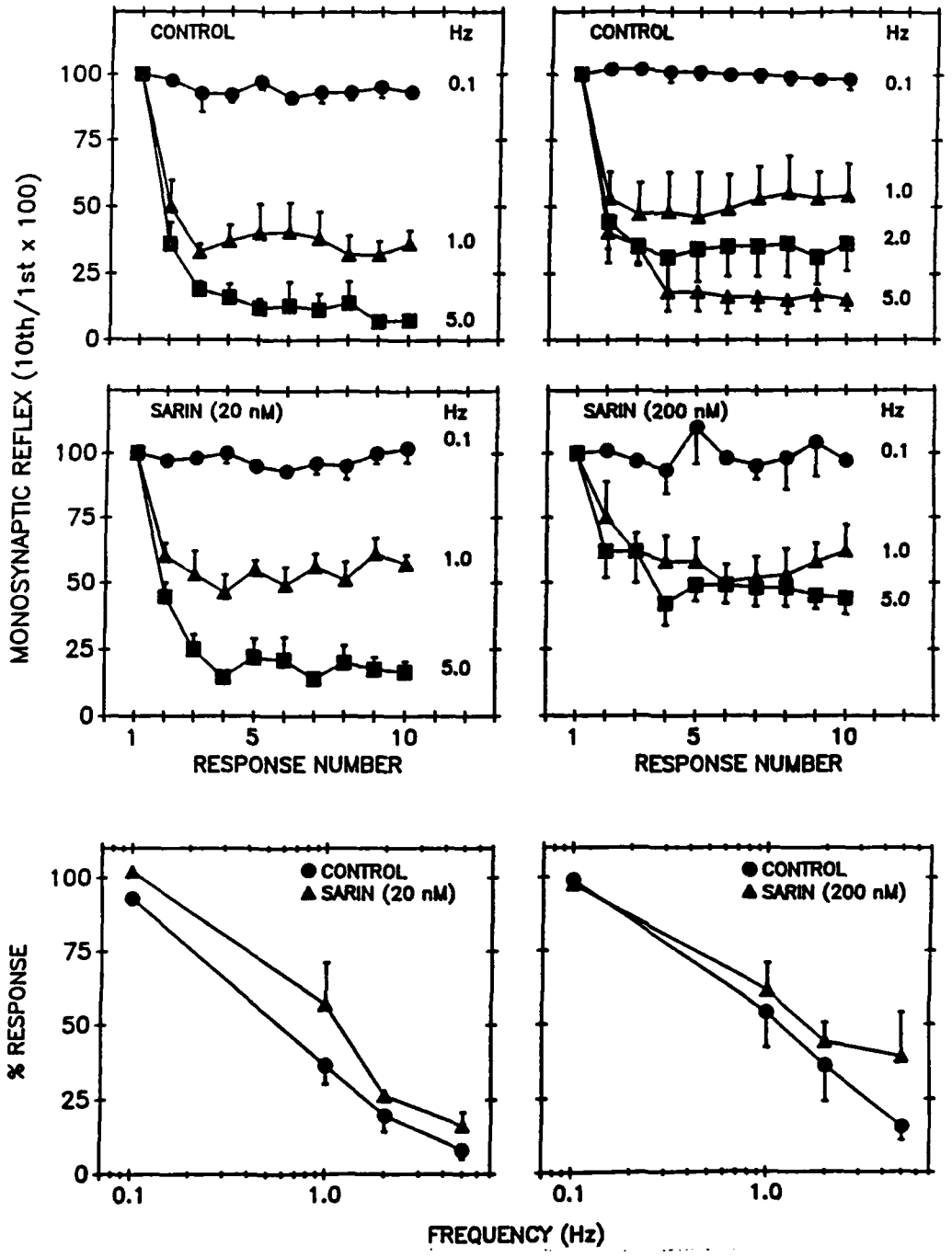

Fig. 12. Modification of frequency-dependent depression of the MSR by sarin. The reflexes were evoked at frequencies from 0.1 to 5 Hz. The degree of frequency-dependent depression before and after sarin is expressed as the ratio of the area of the 10th response to that of the first response in a train of 10 stimuli. The results shown are the mean values (± S.E.M.) obtained from 3 spinal cords at 20 and 200 nM sarin in which each frequency of stimulation was repeated throughout the experiment. The data at 2 Hz in 20 nM sarin was eliminated for clarity of presentation but appears in the summary graph. After exposure to sarin, atropine (200 nM) was added to the toxin-containing solution but failed to have any significant ($P > 0.05$) effect on the response to sarin.

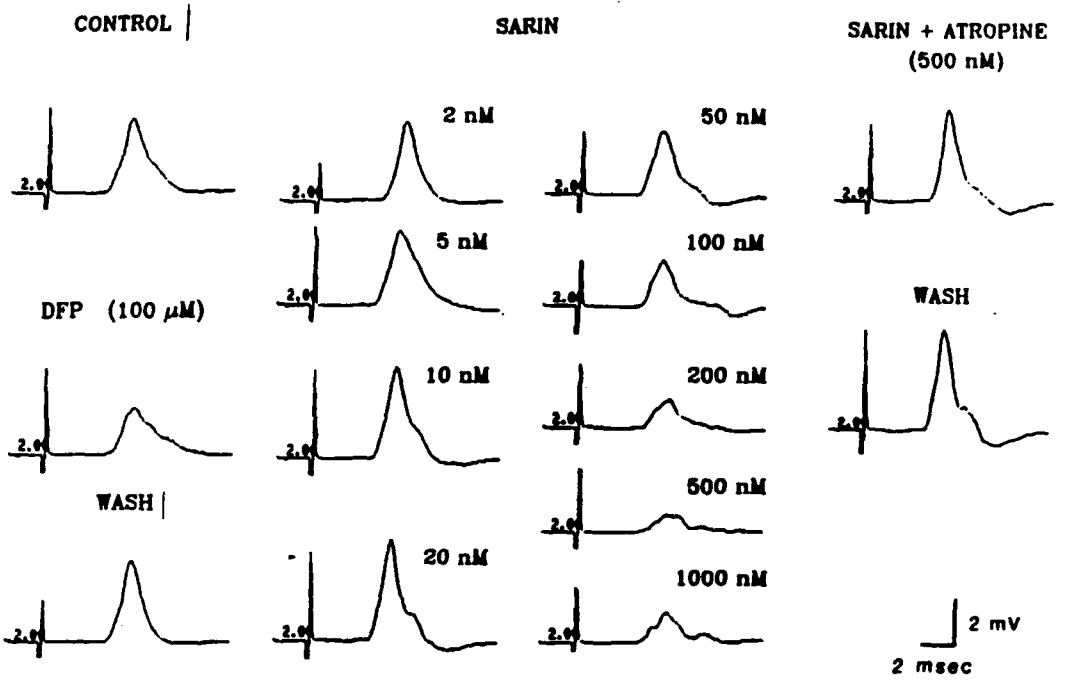

Fig. 13. Inhibition of cholinesterase by DFP does not alter the biphasic action of sarin on the MSR. Control records of the MSR were obtained before and after exposure to DFP (100 μM) for 60 min. The preparation was then washed for 60 min and the reflex had returned to control. The cord was then exposed to progressively increasing concentrations of sarin (2-1000 nM) for 30 min each. After exposure to the highest concentration of sarin, atropine (500 nM) was added to the solution which contained sarin. Subsequently, the cord was washed with normal physiological solution. Each trace is the signal-averaged digitized record of 10 reflexes (0.1 Hz).

C. Alteration of Spinal Monosynaptic Transmission by Carbamates

1. *Physostigmine and Pyridostigmine on the Monosynaptic Reflex*

Neither PHY (0.01-0.1 μM) nor PYR (0.01-0.3 μM) affected the MSR at low concentrations. Increasing the concentration of PHY to 0.3 μM (Figs. 14 and 15) and PYR to 0.6 μM (Fig. 15) significantly ($P < 0.05$) reduced the MSR to 84% and 82% of control, respectively. The subsequent depression of the MSR by either compound was concentration-dependent, with PHY more potent than PYR. For example, at 1 μM PHY, the MSR decreased to about 10% of control (Figs. 14 and 15) while 1 μM PYR depressed the MSR to 55% of control (Fig. 15). At 10 μM PYR the MSR decreased to 35% of control. The concentrations of PHY and PYR which depressed the MSR by 50% (IC_{50}) were about 0.45 μM and 2 μM, respectively.

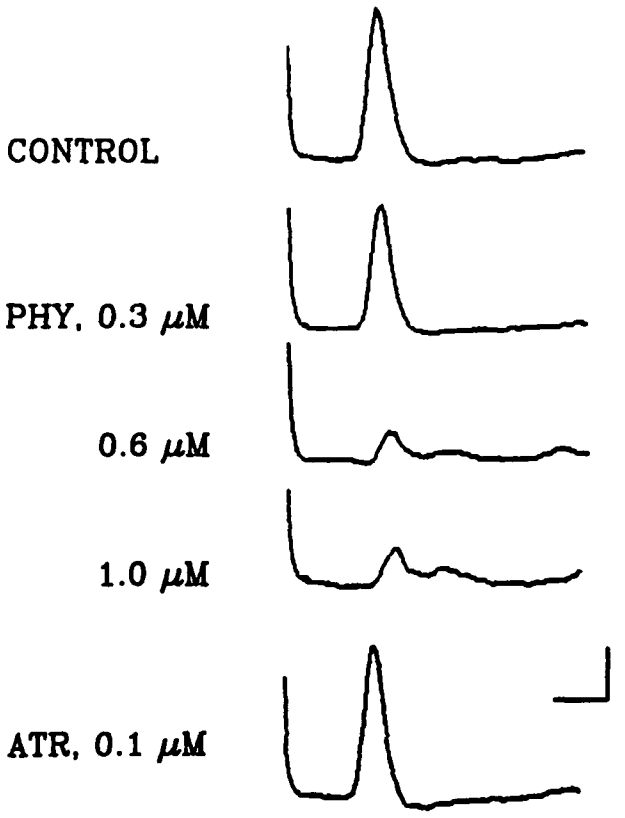

Fig. 14. Effect of physostigmine (PHY) on the MSR. Each trace is a signal-averaged, digitized record of 5 recordings from an L_5 ventral root after stimulating the corresponding dorsal root at 0.1 Hz. Recordings were made every 15 min, after which the concentration of PHY was increased. After exposure to the highest concentration of PHY, the cord was exposed to atropine (ATR) alone and recordings were made after 15 min. The calibrations are 1 mV and 1 msec.

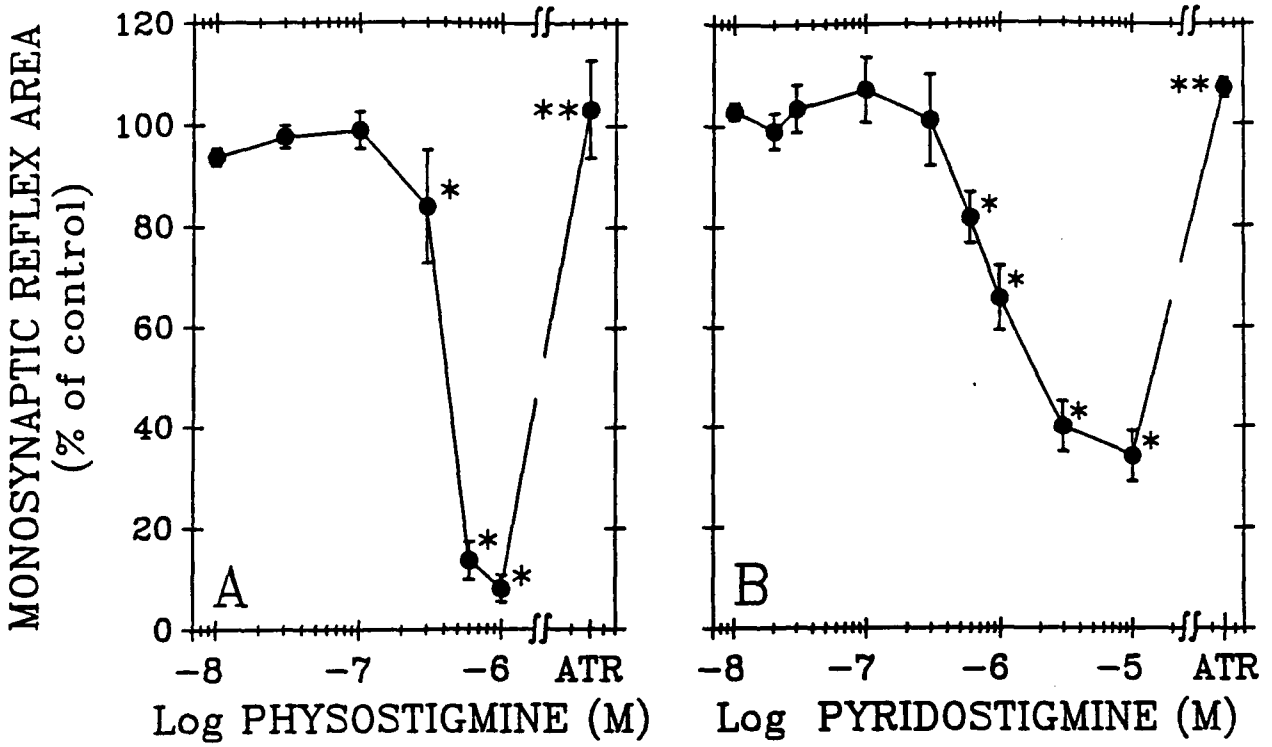

Fig. 15. Dose-response curves for the effect of physostigmine and pyridostigmine on the MSR. Physostigmine (A) and pyridostigmine (B) were applied for 15 min at each concentration, at which time recordings of the MSR were made at a steady-state condition. At the highest concentrations of physostigmine and pyridostigmine, 0.1 μM atropine was applied for 15 min by itself and recordings were made. Note that the response in the presence of atropine after pyridostigmine and physostigmine was significantly ($P < 0.001$; Student's t test) greater than control (**). The values shown are the means ± S.E.M. (n = 3-5 for physostigmine and 4-10 for pyridostigmine). Asterisks (*) indicate a significant difference ($P < 0.05$, Student's t test) between that concentration and control and the concentration above and/or below it.

2. *Reversal of Carbamate-Induced Reflex Depression by Atropine*

The depression of the reflex by the carbamates could be completely antagonized by atropine (0.1 μM), and a potentiation of the MSR could often be observed when either of the carbamates and atropine were present simultaneously (Figs. 14 and 15). The reversal of carbamate-induced depression by atropine was concentration-dependent. After exposure to PHY or PYR (0.6 μM) for 15 min, the cords were simultaneously superfused with atropine (0.01-0.3 μM) + carbamate for an additional 15 min (Fig. 16). At 0.1 μM atropine, the reflex had returned to control levels; raising the concentration of atropine to 0.3 μM in the presence of PHY or PYR resulted in a potentiation of the MSR ($P < 0.01$). The curves for atropine + carbamate were significantly different ($P < 0.05$, ANOVA) from the responses in 0.6 μM PHY or PYR alone (Fig. 16).

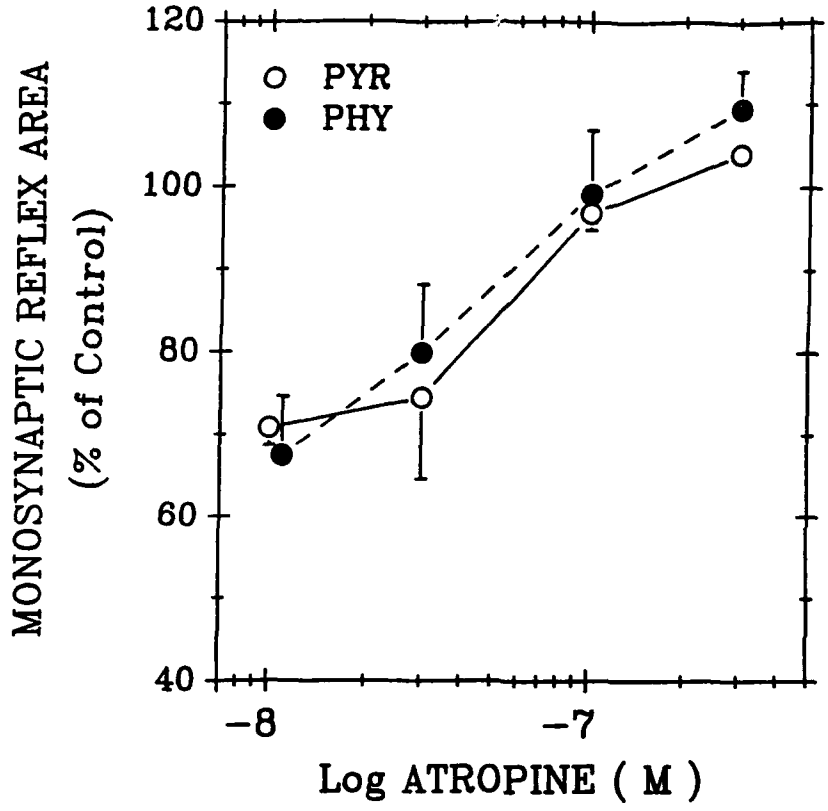

Fig. 16. Concentration-dependent reversal of carbamate-induced depression of the MSR by atropine. The cords were pretreated with 0.6 μM PHY or PYR for 15 min after which the cords were simultaneously exposed to carbamate + atropine. The concentration of atropine was progressively increased from 0.01 to 0.3 μM after 15 min. The values shown are mean $\pm$ S.E.M. (n = 3 for each point with PYR; n = 4 for each point with PHY). There was a significant difference ($P < 0.05$, ANOVA) between each concentration and the response to carbamate alone. The data at 10 nM PHY was offset for clarity of presentation.

3. *Carbamate Pretreatment on Sarin-Induced Depression*

We attempted to determine whether pretreatment with carbamates would afford any protection against segmental depression by irreversible AChE inhibitors *in vitro* as had already been shown *in vivo*. The spinal cords were first exposed for 30 min to one of two concentrations (0.01 and 0.1 μM) of either PYR or PHY. After the pretreatment, the cords were simultaneously exposed to sarin (0.1-0.5 μM) + PHY or PYR for an additional 15 min. Pretreatment with 0.01 μM and 0.1 μM PHY alone for 30 min reduced the MSR to 98.9 $\pm$ 3.2 ($P > 0.05$, Student's t test) and 85.4 $\pm$ 1.0% ($P < 0.05$, Student's t test) of the control, respectively. Pretreatment with 0.01 μM PHY had no beneficial action against sarin-induced depression ($P > 0.05$, Student's t test) (Fig. 17). When different concentrations of sarin were applied to the cords pretreated with 0.1 μM PHY, there was a significantly greater ($P < 0.05$, Student's t test) decrease in the MSR than with sarin alone.

Pretreatment with PYR (0.01 μM) for 30 min had no significant effect ($P > 0.05$, Student's t test) on either the magnitude of the MSR or on the concentration-dependent depression caused by sarin in the presence of PYR (Fig. 18). However, at the highest concentration of sarin (0.5 μM) + 0.01 μM PYR, there was a small, but significant ($P < 0.05$) reduction in the response to sarin when compared to sarin alone (Fig. 18).

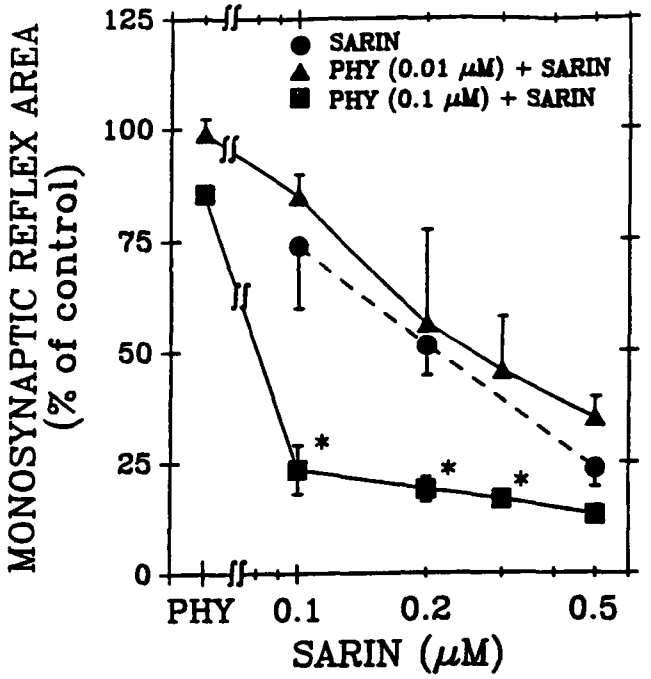

Fig. 17. Physostigmine (PHY) pretreatment fails to protect against sarin-induced depression of the MSR. Cords were exposed to either sarin alone (15 min) or to 0.01 or 0.1 μM PHY for 30 min and then to PHY + sarin for an additional 15 min at each concentration of sarin. Each point is a mean ± S.E.M. (n = 3-4). Asterisks indicate significance ($P < 0.05$) of 0.1 μM PHY pretreatment when compared to sarin alone by Student's t test. Note that the depression observed with sarin was exacerbated at 0.1 μM PHY.

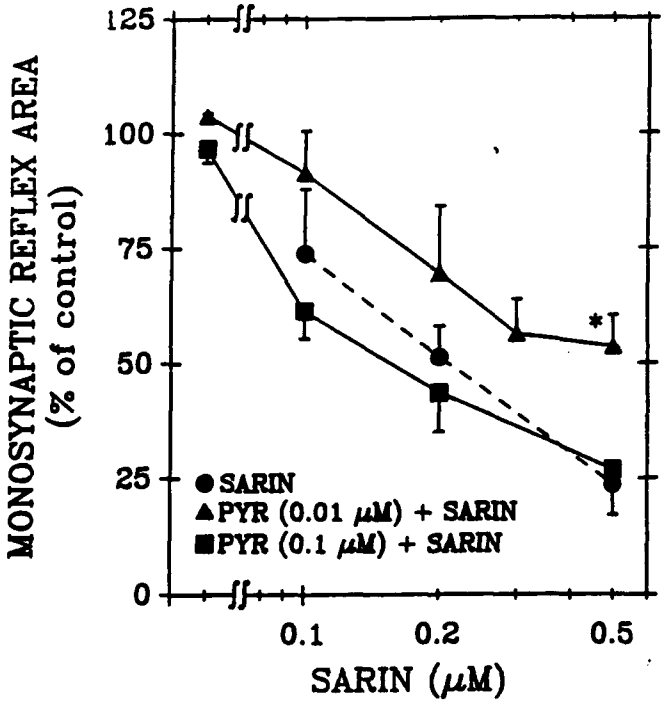

Fig. 18. Pyridostigmine (PYR) pretreatment fails to protect against sarin-induced depression of the MSR. Cords were exposed to either sarin alone (15 min) or to 0.01 or 0.1 μM PYR for 30 min and then to PYR + sarin for an additional 15 min at each concentration of sarin. Each point is a mean ± S.E.M. (n = 3). The asterisk indicates a significant ($P < 0.05$) reduction in depression which occurred at one concentration of sarin in 0.01 μM PYR.

4. *Effect on Spinal Cord Cholinesterase Activity*

Both PHY and PYR produced a concentration-dependent depression of total ChE activity in the spinal cord (Fig. 19). For example, the total ChE activity of the cord in the presence of 0.01 μM PHY was 88.0 $\pm$ 6.6 % of control while at 3 μM it had decreased to 4.4 $\pm$ 0.3 % of control. With PYR, total ChE activity was 98.8 $\pm$ 0.5 % of control at 0.01 μM and 28.8 $\pm$ 1.1 % of control at 3 μM. The IC_{50}'s for PHY and PYR were each about 0.8 μM (Fig. 19).

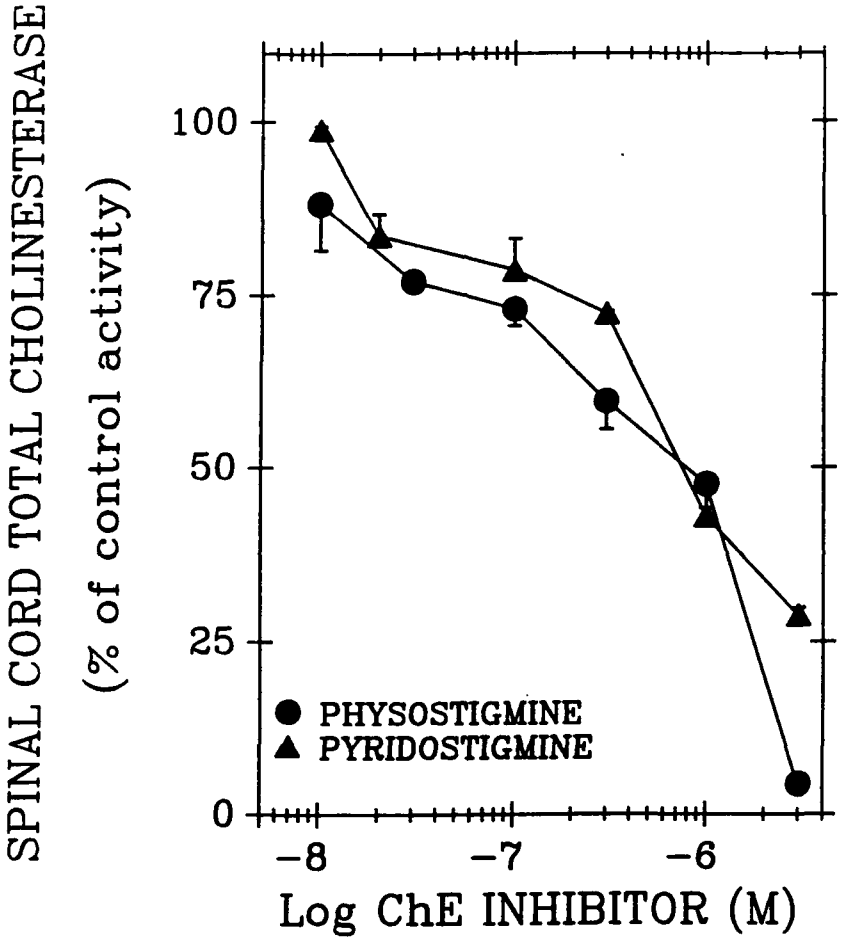

Fig. 19. Concentration-dependent depression of cholinesterase (ChE) activity by carbamates in isolated spinal cords of neonatal rats. Individual spinal cords were exposed to various concentrations of physostigmine or pyridostigmine for 30 min. Estimations of ChE activity were carried out on homogenates of these cords obtained from 8- to 9-day-old rats. The values shown are means $\pm$ S.E.M. of 3 experiments at each concentration of carbamate.

D. Reversal of Organophosphorus-Induced Depression by Oximes

1. *Pralidoxime, Trimedoxime, and Diethyxime on the Monosynaptic Reflex*

None of these oximes alone had a significant effect ($P > 0.05$; ANOVA) on the MSR at concentrations from 0.1 to 10 μM (Figs. 20-22).

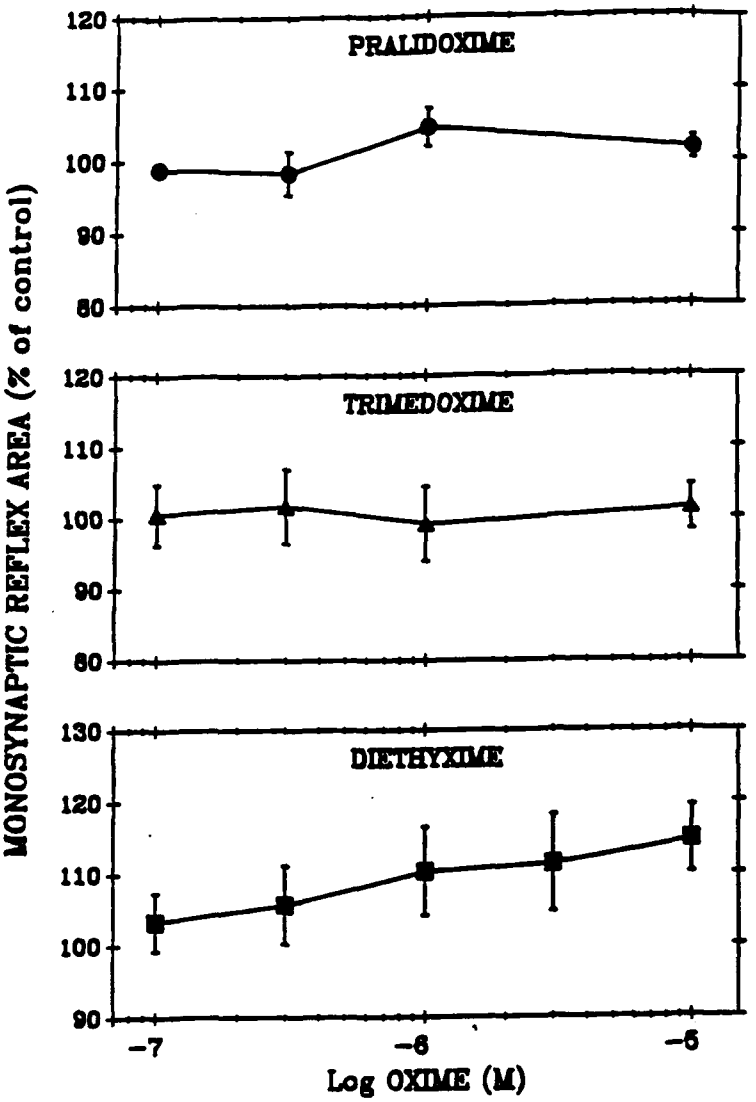

Fig. 20

Fig. 21

Fig. 22

Figs. 20-22. Effect of oximes on the MSR. The cords were superfused for 30 min at each concentration of oxime at which time recordings were made. The values shown are the mean ± S.E.M. (n = 3-5 experiments at each concentration of oxime). There was no significant effect of the oximes on the reflex ($P > 0.05$; ANOVA).

2. *Reversal of Sarin-Induced Depression by Oximes*

Each of the oximes was, however, effective in reversing the depression caused by sarin (Figs. 23-25). At 0.1 μM, sarin depressed the MSR to about 36% of control (n = 10) after exposure for 2 hr (Fig. 20). Subsequent exposure to sarin + varying concentrations of an oxime (0.1-10 μM) resulted in a dose-dependent reversal of OP-induced depression. At 10 μM pralidoxime + 0.1 μM sarin, the reflex had returned to the control value (97% recovery of the reflex) (compare Fig. 20 and Fig. 23). With trimedoxime (10 μM), the recovery was 111% (compare Fig. 21 and Fig. 24) and with diethyxime, 77% (compare Fig. 22 and Fig. 25).

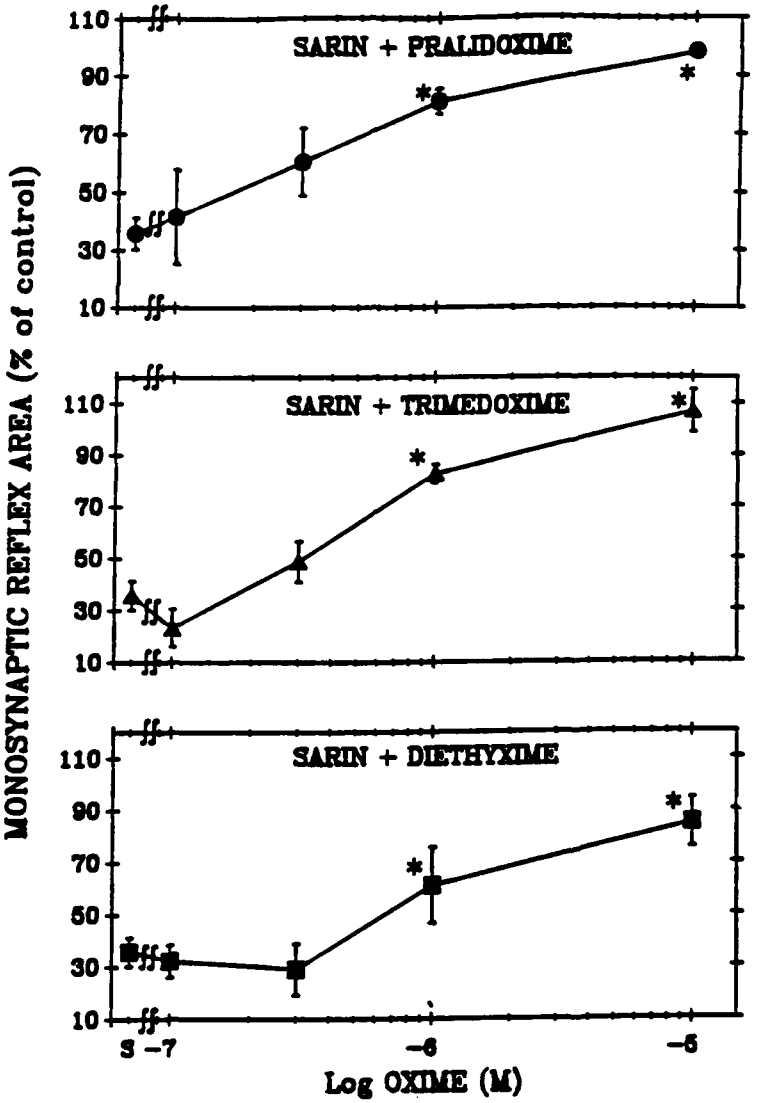

Fig. 23

Fig. 24

Fig. 25

Figs. 23-25. Reversal of sarin-induced depression of the MSR by oximes in cords from neonatal rats. Recordings were made from cords before and after exposure to sarin (S; 0.1 μM) for 2 hr. Cords were then exposed to different concentrations of each oxime + sarin (0.1 μM) for an additional 30 min and recordings were again made. The values shown are the mean $\pm$ S.E.M. (n = 3-5 experiments at each concentration of oxime + sarin; n = 10 for sarin alone). An asterisk indicates a significant difference ($P < 0.05$; ANOVA) between the effect of sarin alone and in the presence of an oxime + sarin.

3. *Effect on Cholinesterase Activity*

Neither pralidoxime, trimedoxime, nor diethyxime (10 μM) had a significant ($P > 0.05$) effect on spinal cord AChE activity (109.8 $\pm$ 7.7, 107.3 $\pm$ 1.8, 114 $\pm$ 6.2% of control, respectively; n = 3 for each). When the spinal cord was exposed to sarin (0.1 μM) for 2 hr, the AChE activity was reduced ($P < 0.001$) to 52.6 $\pm$ 1.0% of control (n = 9). Spinal cord AChE activity in the presence of sarin (0.1 μM) + pralidoxime, trimedoxime, or diethyxime (10 μM) for 30 min was 55.5 $\pm$ 1.8%, 48.3 $\pm$ 1.5% and 45.2 $\pm$ 1.7% of control (n = 3 each), respectively, values which were not significantly different ($P > 0.05$) from sarin alone.

E. Interaction of TRH with OP-Induced Monosynaptic Depression

1. *Reversal of OP-Induced Monosynaptic Depression by TRH*

Both DFP and sarin depressed the MSR. At 100 and 500 μM DFP, the area of the MSR was reduced to 40 and 18% of control (Fig. 26). After exposure to 100 μM DFP, the cords were simultaneously exposed to varying concentrations of TRH (range 30-1000 nM). Maximal reversal of the depression caused by DFP occurred at 100 nM TRH. The reflex attained 95% of its control value and was not significantly different ($P > 0.1$) from pre-DFP values. No further increase in the magnitude of the MSR occurred even when the concentration of TRH was increased to 1 μM (Fig. 26). The ability of TRH to reverse the effect of 500 μM DFP was also observed. However, the maximal reversal to nearly 75% of control occurred at 300 nM TRH.

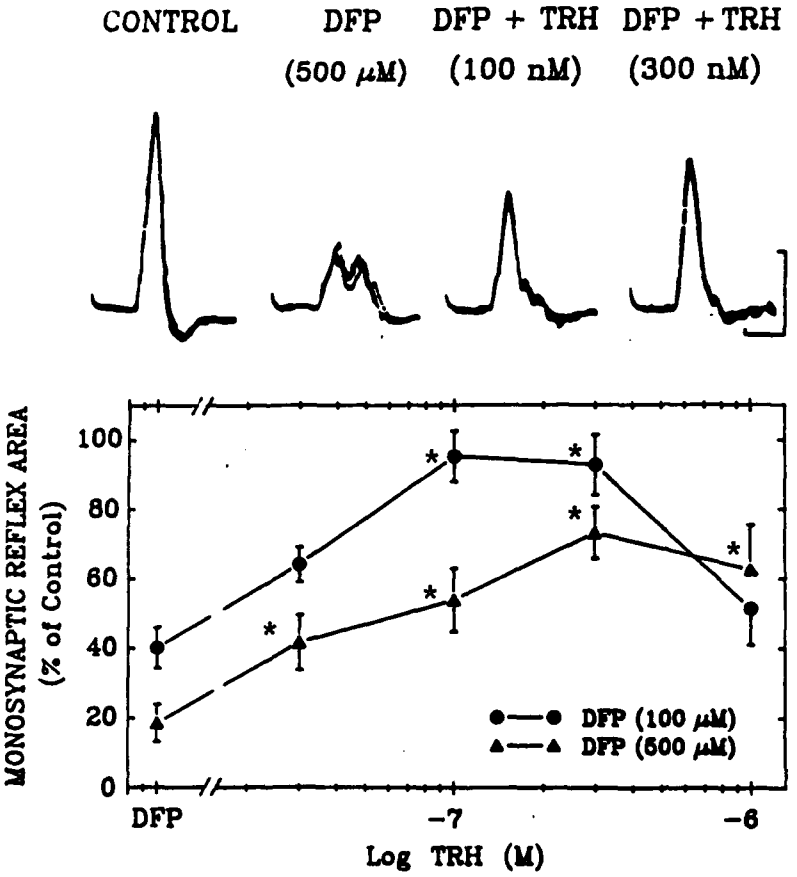

Fig. 26. Reversal of DFP-induced depression of the MSR by thyrotropin-releasing hormone (TRH). The top panel shows five superimposed tracings of the MSR recorded from an L_5 ventral root after stimulating the corresponding dorsal root at 0.1 Hz. The cord was then exposed to DFP (500 μM) for 15 min and then to DFP + TRH for 15 min each at progressively higher concentrations. The tracings shown at 15 min after applying or increasing the concentration of TRH represent a steady-state condition obtained within 5 min of applying or raising the TRH concentration. Lower panel shows the dose-response curve for the effect of TRH on the MSR before and after exposure to DFP for 15 min. The values shown are the mean ± S.E.M. where: n = 10 at 100 μM DFP; n = 5 at 500 μM DFP; n = 3-5 at each concentration of DFP + TRH. Note the maximal reversal of DFP-induced depression occurs at 100 nM TRH in the presence of 100 μM DFP. Vertical calibration = 2 mV; horizontal calibration = 5 msec. The asterisk (*) indicates a significant difference ($P < 0.05$, ANOVA) between the maximal depression caused by either 100 or 500 μM DFP and the response in the presence of TRH.

The depression caused by sarin could also be reversed by simultaneous application of TRH (Fig. 27). Sarin (100 nM) depressed the MSR to about 46% of control. The maximal reversal of sarin-induced depression occurred at 1 μM TRH (in the presence of sarin) where the MSR reached more than 95% of control, a value not significantly different ($P > 0.1$) from pre-sarin controls. Increasing the concentration of TRH above 1 μM did not result in a further reversal of depression (Fig. 27).

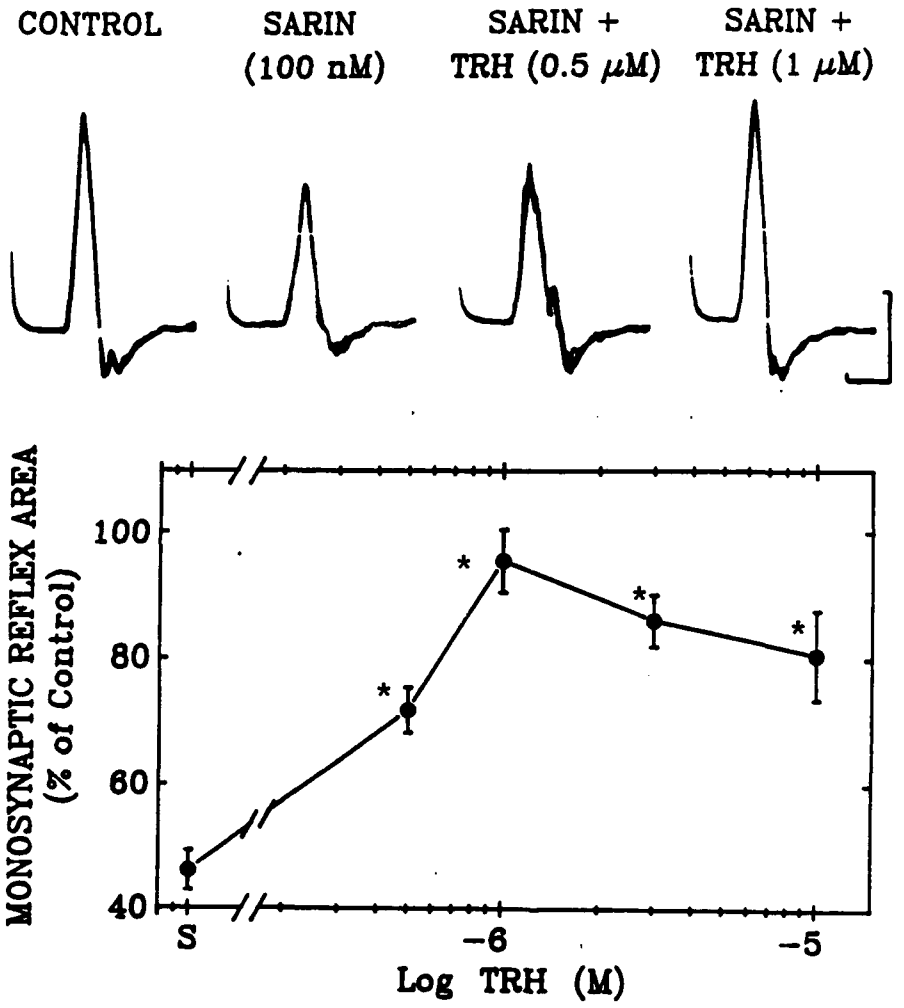

Fig. 27. Reversal of sarin-induced depression of the MSR by thyrotropin-releasing hormone (TRH). The top panel shows 5 superimposed tracings of the MSR before and after exposure to sarin (100 nM) for 120 min and then in the presence of TRH + sarin for 15 min each at progressively higher concentrations. The lower panel shows the effect of 100 nM sarin (S) on the reflex and the dose-response curve for the reversal of sarin-induced depression. TRH was applied for 15 min at each concentration, at which time recordings were made at a steady-state condition. The values shown are the mean ± S.E.M. (n = 4-5). Asterisks (*) indicate a significant difference ($P < 0.05$, ANOVA) between the effect of sarin alone (S) and in the presence of TRH + sarin. Maximal reversal of sarin-induced depression occurred at 1 μM TRH. Vertical calibration = 2 mV; horizontal calibration = 5 msec.

2. *TRH and Atropine on the Monosynaptic Reflex*

As previously shown, TRH produced a concentration-dependent increase in the area of MSR in cords from male rats.[37] A small but significant ($P < 0.05$) increase in the magnitude of the MSR occurred at 30 nM TRH with maximal potentiation at 1000 nM TRH (Fig. 28). Atropine alone had no effect ($P > 0.05$) on the magnitude of the MSR at 1 μM (103 $\pm$ 1.9 % of control; n = 9) and did not alter the magnitude of potentiation of the MSR induced by 100 nM or 1000 nM TRH (Fig. 28).

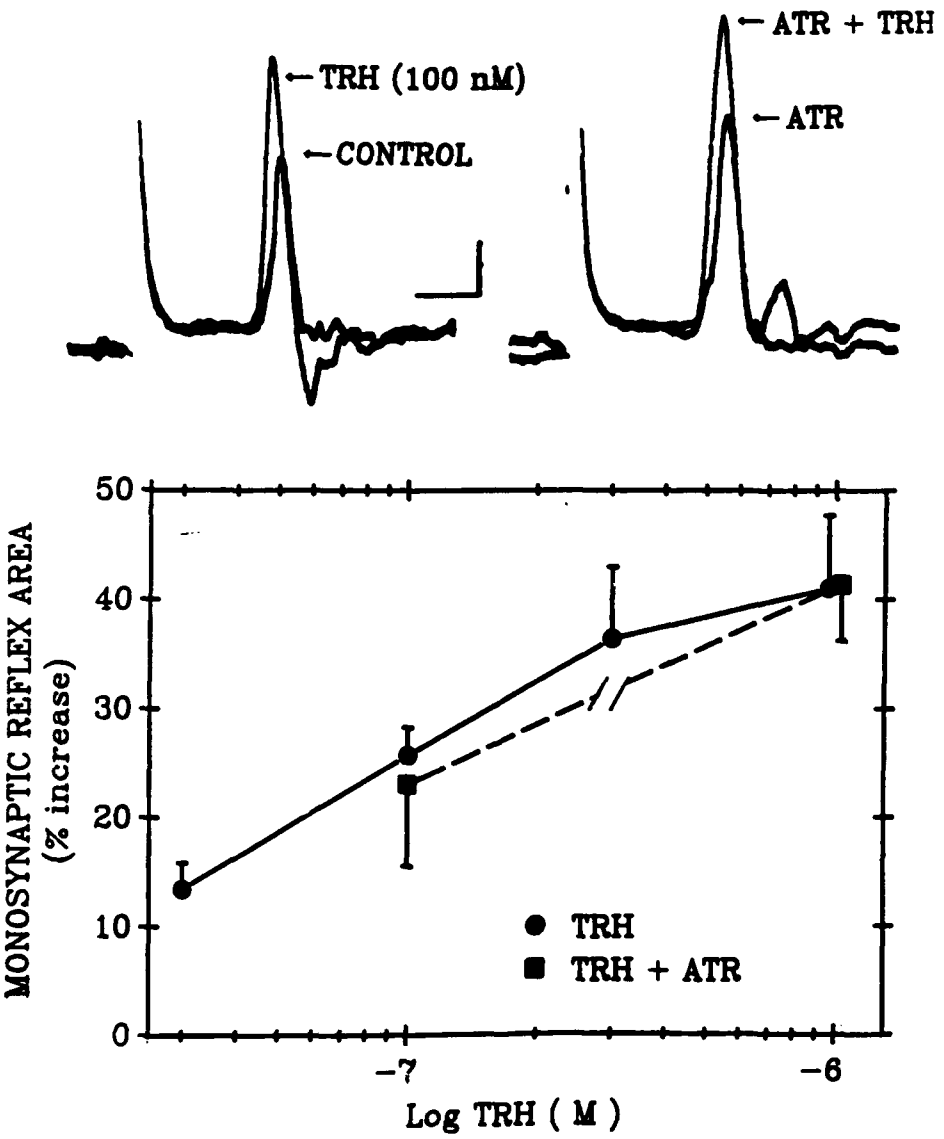

Fig. 28. Lack of effect of atropine on TRH-induced potentiation of the MSR. The upper panel shows two sets of superimposed reflexes obtained from spinal cords of 8-day neonatal male rats. The traces on the left illustrate the potentiation caused by TRH (100 nM) and the traces on the right illustrate the effect of TRH (100 nM) in the presence of atropine (1 μM). The lower panel shows the dose-response curve for potentiation of the MSR by TRH (n=7) and the response to 100 nM (n=3) and 1000 nM (n=6) TRH in presence of 1 μM atropine. The magnitude of potentiation by TRH (100 nM) was not significantly different ($P > 0.05$) in presence or absence of atropine. The values are expressed as the mean $\pm$ S.E.M.. Vertical calibration = 1 mV; horizontal calibration = 2 msec.

3. *TRH and OPs on Cholinesterase Activity*

DFP and sarin inhibited ChE activity in supernatants of the whole spinal cord. When exposed to 100 μM DFP or 100 nM sarin for 15 and 120 min, respectively, ChE activity was reduced by about 75% and 60%, respectively. The inhibition of enzyme activity by DFP and sarin persisted even in the presence of TRH at concentrations which reversed MSR depression (Fig. 29).

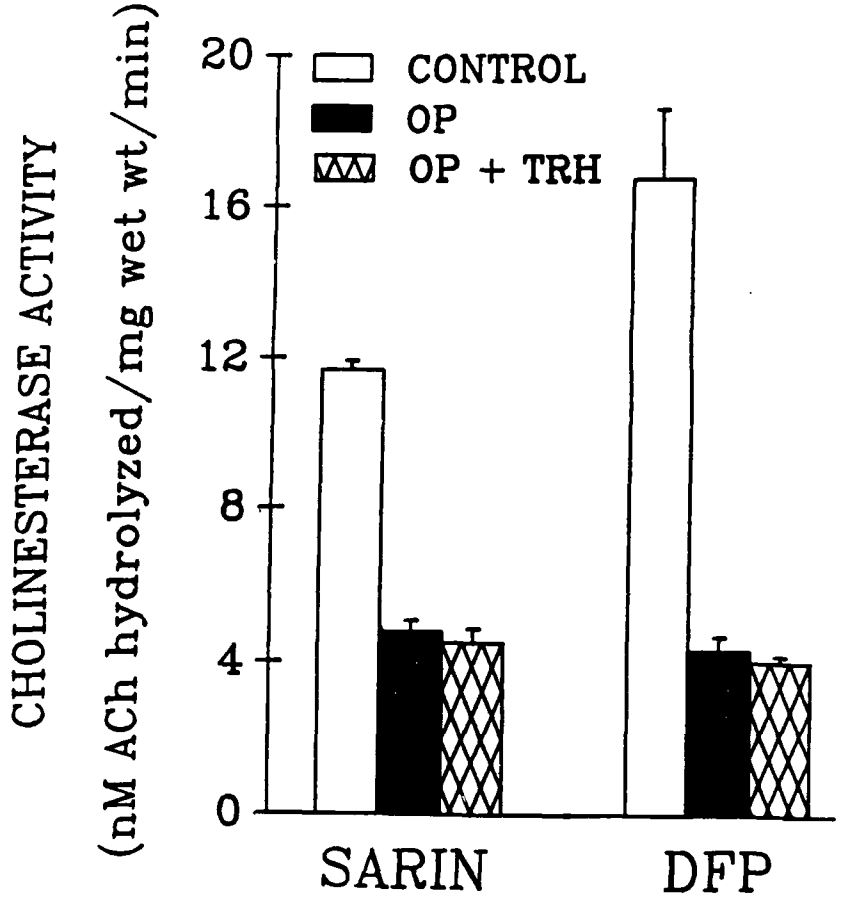

Fig. 29. Effect of sarin, DFP and thyrotropin-releasing hormone (TRH) on spinal cord cholinesterase (ChE) activity. The histograms show ChE activity expressed as nM of acetylcholine (ACh) hydrolyzed per mg wet weight per min in supernatants from whole spinal cord of 8- to 9-day old male rats. The spinal cords were exposed to sarin (100 nM) or DFP (100 μM) for 120 and 15 min, respectively. At this time, sarin and DFP had significantly inhibited ($P < 0.001$) the ChE activity by 60 and 75%, respectively. Subsequent exposure of the cords to TRH (300 nM) + DFP or sarin had no effect on residual ChE activity. The values are expressed as mean $\pm$ S.E.M. (n=6).

F. Effect of Sarin on Spinal Inhibition

1. *Characteristics of Inhibition in the Isolated Spinal Cord*

Test stimuli applied to a dorsal root evoked a MSR in the corresponding ventral root with a latency of about 4 msec and a rise time of less than 1 msec (Fig. 30). These values were in

agreement with previously published results.[39,117,135] Maximal inhibition occurred at C-T intervals of 7 msec when the reflex was reduced to about 40% of control (Figs. 30 and 31). As the C-T interval was lengthened, the amplitude of the reflex increased such that at C-T intervals of 20-70 msec, the reflex had reached a plateau at about 70% of control. The early (7 msec) and late (20-70 msec) phases of inhibition correspond to the strychnine-sensitive, glycine-mediated, and bicuculline-sensitive, GABA-mediated inhibitions in the neonatal cord, respectively.[39]

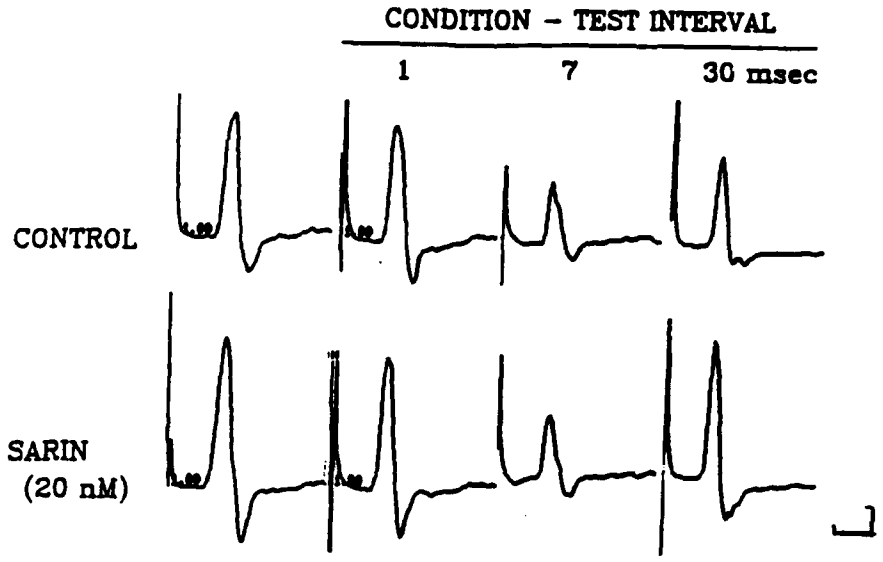

Fig. 30. Typical effect of sarin on the late phase of spinal inhibition. Digitized records of single MSRs obtained under control conditions and at varying conditioning-test intervals before and after a facilitating concentration of sarin. The traces illustrate the early phase of inhibition occurring at 7 msec (strychnine-sensitive component) and the later phase of inhibition (bicuculline-sensitive component) occurring at 30 msec. Note the increase in magnitude of the MSR in the presence of sarin and reduction of the later phase of inhibition. Calibration, 1 mV; 5 msec.

2. *Effect of Sarin on Spinal Inhibition*

When the cords were exposed to concentrations of sarin (3-20 nM) which are known to increase the amplitude of the reflex, the magnitude of the reflex increased, but the early phase of inhibition was unaffected (Figs. 30-32). The late phase of inhibition was, however, progressively reduced in magnitude such that at 20 nM sarin, the late phase of inhibition was completely absent (Fig. 31). As the concentration of sarin was increased above 20 nM (*i.e.*, to depressant concentrations), the amplitude of the MSR decreased concomitant with a loss and then enhancement of the bicuculline-sensitive phase of inhibition. There appeared to be no effect on the early, strychnine-sensitive phase of inhibition.

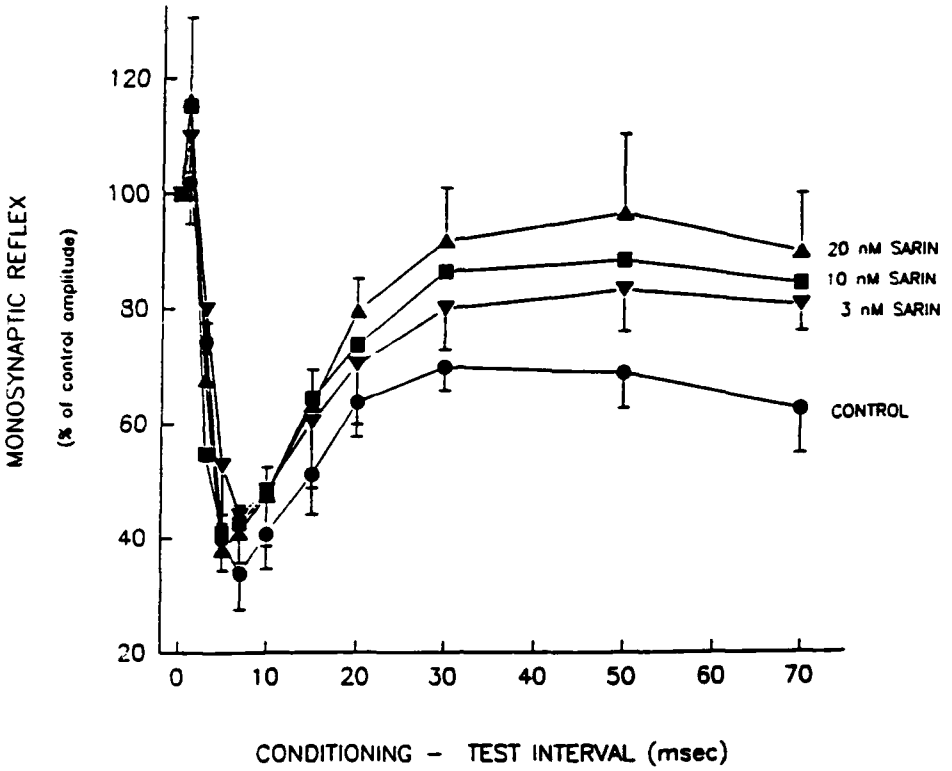

Fig. 31. Effect of sarin (3-20 nM) on the time course of inhibition of the MSR. The graph shows the mean response (± S.E.M.) of 4 control and 4 experiments with sarin at each concentration. The values in the presence of sarin at conditioning-test intervals of 20-70 msec were significantly different ($P < 0.05$, ANOVA) than control. The error bars at 10 nM were removed for clarity of presentation but did not exceed those at 3 nM sarin.

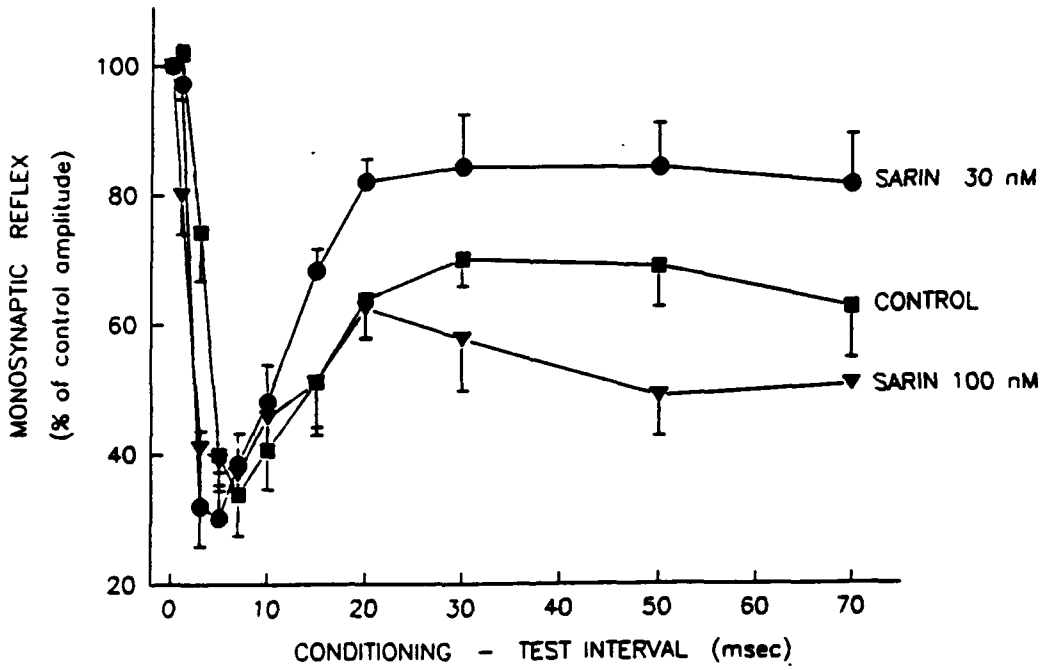

Fig. 32. Effect of sarin (30 and 100 nM) on the time course of inhibition of the MSR. The graph shows the mean response (± S.E.M.) of 4 control and 4 experiments with sarin at each concentration. The values in the presence of 30 nM sarin at conditioning-test intervals of 20-70 msec were significantly greater ($P < 0.05$, ANOVA) than control, while those at 100 nM sarin were significantly lower ($P < 0.05$, ANOVA) than control. The control curve is the same as in Fig. 31.

The concentration dependence of the block of inhibition is shown in Fig. 33 at a C-T interval of 50 msec. At 3-20 nM sarin, the bicuculline-sensitive inhibition was progressively blocked, but at higher concentrations inhibition was at first reduced and then enhanced.

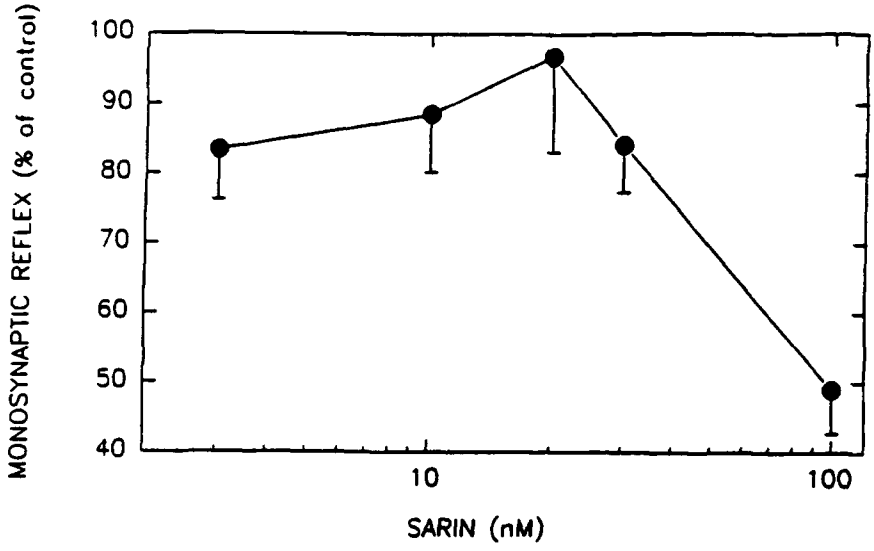

Fig. 33. Concentration-dependent block of bicuculline-sensitive inhibition by sarin. The degree of block decreased progressively from 3-20 nM sarin but was greater at higher concentrations. The effect was determined at condition-test intervals of 50 msec. Each point is the mean of 3-5 observations.

G. NMDA and *non*-NMDA Receptors in Reflex Spinal Transmission

1. *Effect of Mg^{2+} on Mono- and Polysynaptic Reflexes*

Stimulation of an L_{3-5} dorsal root in the absence of Mg^{2+} ions evoked two temporally distinct reflex potentials, with short and long latencies, in the corresponding ventral root which are apparently of monosynaptic and polysynaptic origin, respectively. The mean amplitude and latency of the MSR were 4.5 ± 0.3 mV and 5.6 ± 0.1 msec, and the amplitude and latency for the PSR were 1.5 ± 0.1 mV and 14.1 ± 0.5 msec (n = 36), respectively. The MSR was relatively resistant to an increase in the $[Mg^{2+}]_o$ while the PSR was markedly depressed when Mg^{2+} was added to the superfusate (Fig. 34A). The onset of the reflexes' depression after raising the $[Mg^{2+}]_o$ from zero to 1.3 mM occurred in 2-5 min, reached a plateau in 10-15 min, and usually returned to the control level 15-30 min after rinsing in a Mg^{2+}-free solution. The decrease in magnitude of the MSR by Mg^{2+} occurred initially in a concentration-dependent manner, but with a maximum reduction of about 30% at 1.3-3 mM (Fig. 34). On the other hand, the depression of the PSR by Mg^{2+} was concentration-dependent with 50% inhibition (IC_{50}) at 185 μM, and complete inhibition occurred at 1.3 mM Mg^{2+} (Fig. 34B).

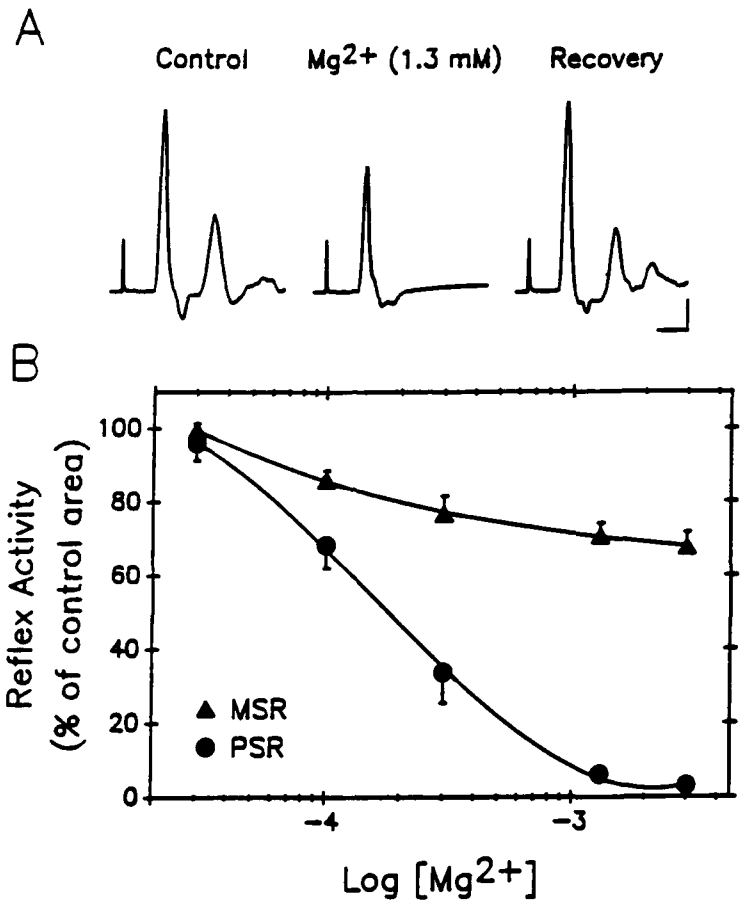

Fig. 34. Differential sensitivities of MSR and PSR to magnesium ions. The spinal cords were isolated in and initially superfused with a Mg^{2+}-free physiological solution. The traces in (A) are signal-averaged records of 10 successive responses in a Mg^{2+}-free solution (Control), 20 min after raising the Mg^{2+} concentration to 1.3 mM and 30 min after rinsing in Mg^{2+}-free solution (Recovery). The calibrations are 1 mV and 5 msec. The concentration-response curves for the depression of the monosynaptic (MSR) and polysynaptic (PSR) reflexes by Mg^{2+} are shown in (B). Each point is the mean ± S.E.M. of 3 to 6 separate experiments in different cord preparations.

2. *Effect of Mg^{2+} on Reflex Activity*

Stimulation of the dorsal root resulted in a response from the ventral root whose waveform was dependent on the [Mg^{2+}] (Table 2). The amplitude of the MSR increased nearly 10% in the absence of Mg^{2+}, while the area decreased nearly 8% and the latency decreased by about 12%. A PSR was apparent in the absence of Mg^{2+} that was lower in amplitude, similar in area, and longer in latency than the MSR in the absence of Mg^{2+}.

TABLE 2

Characterization of the reflex waveform in the presence and absence of Mg^{2+} in spinal cords from neonatal rats, *in vitro*

Values are means ± S.E.M. of 3-6 observations; the responses to 1.3 mM Mg^{2+} were observed 30-60 min after exposure following cord isolation in a Mg^{2+}-free solution.

	Mg^{2+}-free		Mg^{2+} (1.3 mM)
	MSR	PSR	MSR
Amplitude (mV)	4.8 ± 0.4	2.7 ± 0.2	4.4 ± 0.8
Area (mV • ms)	5.7 ± 0.3	5.3 ± 0.7	6.1 ± 1.3
Latency (ms)	4.3 ± 0.3[a]	8.8 ± 0.5	4.9 ± 0.4

[a] $P < 0.05$ with respect to 1.3 mM Mg^{2+}.

3. *Effects of NMDA antagonists on Reflex Activity*

The MSR and PSR were depressed by the competitive NMDA receptor antagonists APV and AP7 in a concentration-dependent manner (range 0.3-10 μM) (Figs. 35 and 36). While the time course of depression of both reflexes by APV or AP7 was similar (onset, 3-8 min; peak effect, 10-20 min; recovery, within 30 min), these agents selectively depressed the PSR (IC_{50} = 2.6 μM for APV and 1.7 μM for AP7, respectively), while causing a small depression of the MSR (Figs. 35 and 36). For example, during the exposure to 10 μM APV or AP7, the MSR only decreased by about 20%, while the PSR was completely blocked. No further decrease in the MSR was observed, even when the concentration of APV or AP7 was increased. At 30 μM APV, the MSR was 76.8 ± 2.8% and the PSR was 0.7 ± 0.7% of control (n = 3); at 30 μM AP7, the MSR was 76.0 ± 7.7% and the PSR was 2.0 ± 2.0% of control (n = 3).

Experiments thus far have revealed that the PSR and a fraction (≈30%) of the MSR are comparably sensitive to the $[Mg^{2+}]_o$ and to NMDA antagonists. Therefore, we were interested in directly comparing the effect of Mg^{2+} removal and APV on the magnitude of the reflexes. Spinal cords were prepared and stabilized in a normal physiological solution which contained 1.3 mM Mg^{2+}. After recordings were obtained, the preparations were superfused in a Mg^{2+}-free physiological solution for 45 min and subsequently exposed to 10 μM APV in the absence of Mg^{2+} for 20 min. In the presence of a normal (1.3 mM) concentration of Mg^{2+}, only the MSR was clearly apparent -- the PSR was only of negligible magnitude (Table 3). During superfusion with the Mg^{2+}-free physiological solution, there was a small increase (18%) in the magnitude of the MSR concomitant with the appearance of the PSR (Table 3). The augmentation of both reflexes disappeared when these cords were subsequently exposed to APV in the absence of $[Mg^{2+}]_o$ (Table 3) or when the original level of Mg^{2+} (1.3 mM) was restored (data not shown, but see for example Fig. 34A).

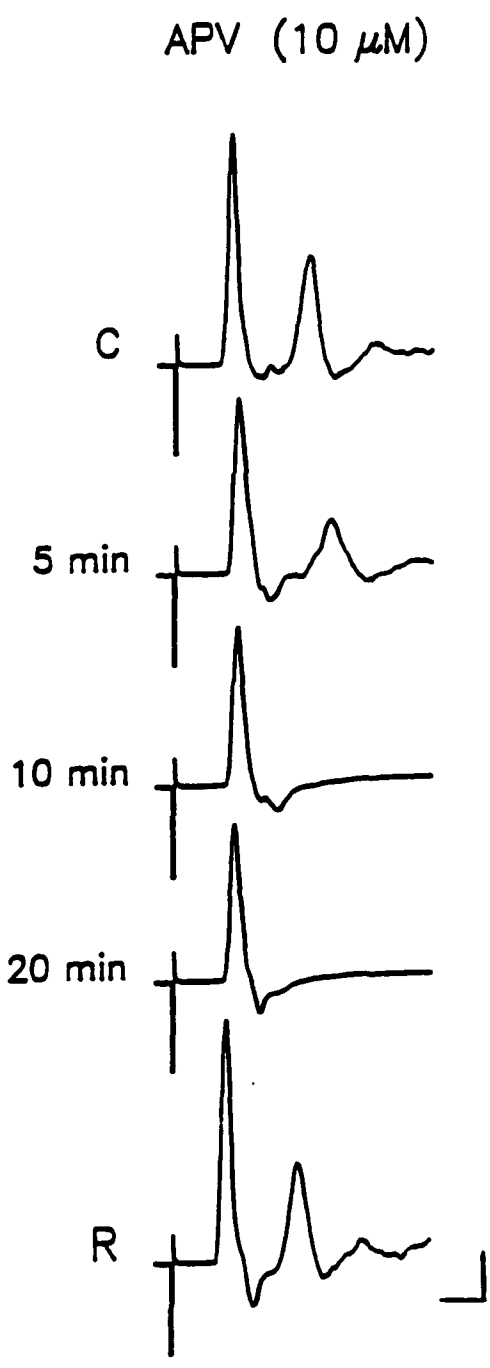

Fig. 35. Effects of the NMDA receptor antagonist APV on MSRs and PSRs. The spinal cords were superfused with a Mg^{2+}-free physiological solution throughout the experiment. After the control (C) recordings were obtained, the spinal cords were exposed to APV (10 μM) for 20 min and then washed in drug-free physiological solution for 30 min, to allow recovery (R). The traces are signal-averaged records of 10 successive responses obtained in the same preparation. The calibrations are 1 mV and 5 msec.

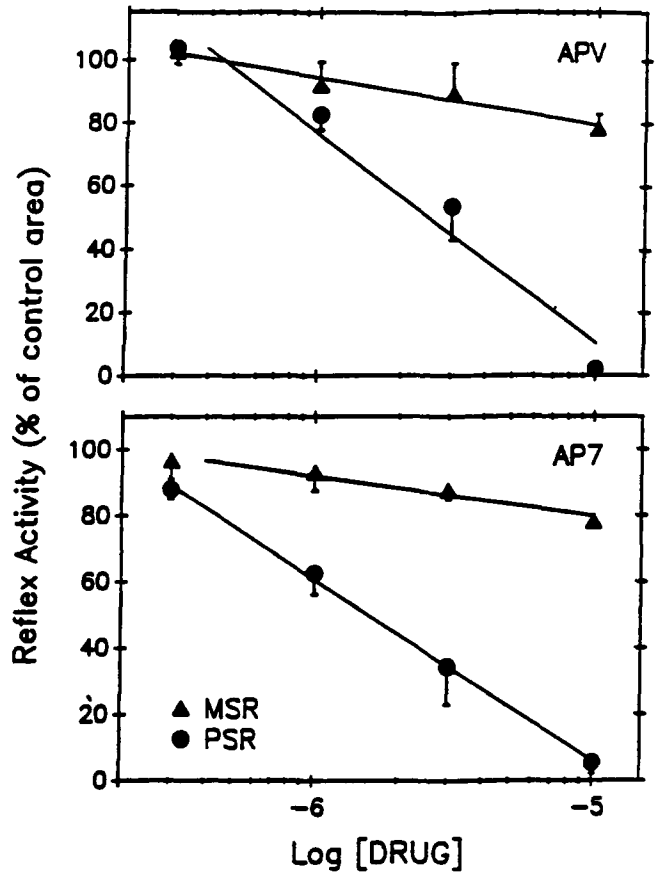

Fig. 36. Differential depression of the MSRs and PSRs by the NMDA receptor antagonists APV and AP7. The spinal cords were superfused with a Mg^{2+}-free physiological solution throughout the experiment. Each concentration of APV or AP7 was applied for 20 min. The points indicate the mean ± S.E.M. of 3 or 4 separate experiments in different cord preparations.

TABLE 3

Augmentation of the monosynaptic and polysynaptic reflexes by removal of Mg^{2+} and its reversal by 2-amino-5-phosphonovalerate (APV) in isolated spinal cords from neonatal rats *in vitro*[a]

The spinal cords were prepared and stabilized in a normal physiological solution containing 1.3 mM Mg^{2+} (control), then exposed to Mg^{2+}-free bathing medium for 45 min and subsequently to APV (10 μM) in the absence of Mg^{2+} for 20 min.

Treatment	Reflex Area (mV·msec)	
	Monosynaptic	Polysynaptic
Control (1.3 mM Mg^{2+})	4.9 ± 0.4	0.2 ± 0.1
Mg^{2+}-free	5.8 ± 0.5*	1.9 ± 0.3**
Mg^{2+}-free + APV (10 μM)	4.5 ± 0.2	0.2 ± 0.1

[a] Values presented are means ± S.E.M. of three separate experiments in different spinal cord preparations.

* $P < .05$, statistically significant as compared to control (paired Student's *t*-test).

** $P < .01$, statistically significant as compared to control (one-way ANOVA and Dunnett's test).

4. *Effect of DNQX on the Mono- and Polysynaptic Reflexes*

In order to observe the effect of DNQX on the *non*-NMDA-mediated component of the MSR, APV, dizocilpine, or Mg^{2+} was applied first at concentrations known to selectively and completely block NMDA-mediated reflex activity. The addition of APV (10 μM) to the Mg^{2+}-free superfusate reduced the area of the MSR to 85% of control and abolished the PSR (Fig. 37). Both dizocilpine (10 μM) and Mg^{2+} (1.3 mM) also reduced the area of the MSR by $\approx$ 15% and abolished the PSR (Fig. 37; see also Fig. 39). In the presence of APV (10 μM) or dizocilpine (10 μM), DNQX depressed the MSR in a dose-dependent manner, with complete block occurring at 3 μM (Fig. 38). However, when the APV was removed from the superfusate with 3 μM DNQX still present, reflex activity returned and the areas of the MSR and PSR were restored to 71% and 65% of control, respectively. Similarly, in Mg^{2+}-free media, 5 μM DNQX depressed the MSR and PSR to 72% and 54% of control, respectively; yet in the presence of 1.3 mM Mg^{2+}, 5 μM DNQX completely blocked all reflex activity (Fig. 39). The DNQX-induced reflex depression was reversed in both the absence and presence of Mg^{2+} by washing with drug-free superfusate for 60 min (Fig. 39).

The increase in potency of DNQX in the presence of either APV (10 μM) or Mg^{2+} (1.3 mM) was quantified (Fig. 38). At a physiological concentration of Mg^{2+} (1.3 mM), DNQX depressed the MSR in a dose-dependent manner with an IC_{50} of 0.70 ± 0.08 μM. Similarly, in a Mg^{2+}-free superfusate with APV (10 μM) present, DNQX had an IC_{50} of 0.93 ± 0.08 μM

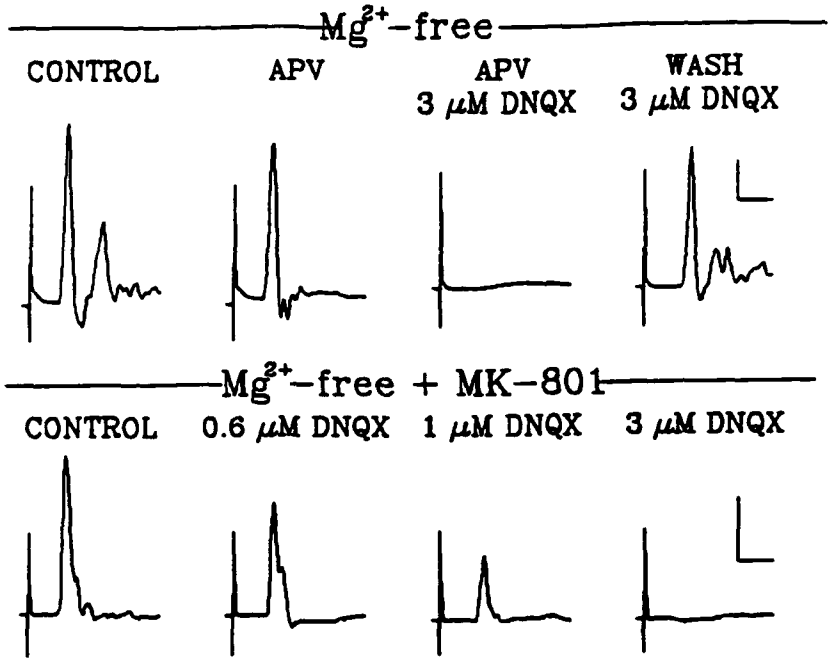

Fig. 37. Effect of NMDA blockade on the effect of DNQX on mono- and polysynaptic reflexes. The traces are signal-averaged records of 5 successive reflexes. The response in Mg^{2+}-free conditions before exposure to dizocilpine (MK-801) is not shown. The first peak after the stimulus artifact is the MSR, and the second is the PSR. Calibrations = 1 mV; 5 msec.

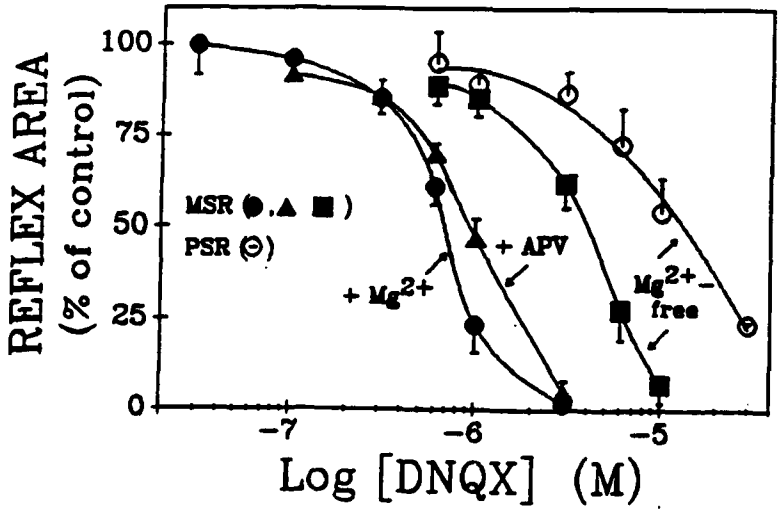

Fig. 38. Dose-response relationship for DNQX on the MSR and PSR and the influence of NMDA antagonism. Each point is the mean ± S.E.M. of 3-8 observations. Each observation for a given concentration was from a different cord; data for up to 6 concentrations may have come from one cord by applying progressively larger concentrations without wash between applications. The control response was defined according to appropriate conditions (*e.g.*, the response in the presence of 10 μM APV was defined as control for the DNQX dose-response curve in the presence of 10 μM APV).

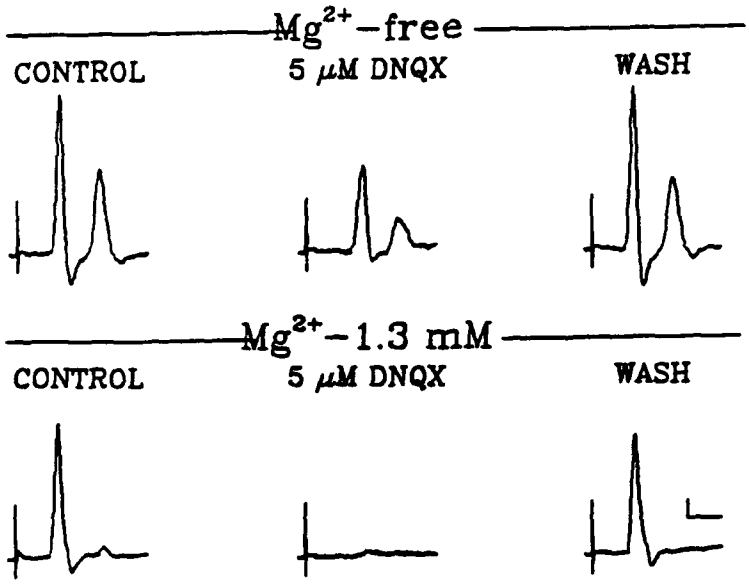

Fig. 39. Effect of Mg^{2+} on the potency of DNQX-mediated depression of the mono- and polysynaptic reflexes. The responses shown are signal-averaged traces of five successive reflexes in the absence and presence of Mg^{2+}: before; 30 min after exposure to DNQX (5 μM); and 60 min after wash with drug-free superfusate. The first and second peaks after the stimulation artifact are, respectively, the MSR and PSR. Calibration: 1 mV, 5 ms.

for the MSR. In a Mg^{2+}-free superfusate where NMDA receptors are functional, the IC_{50}s of DNQX for the MSR and PSR were 13.6 $\pm$ 4.3 μM and 3.9 $\pm$ 0.4 μM, respectively. The potency of DNQX on the MSR therefore increased 15- to 20-fold when NMDA receptors were inactive. The IC_{50}s of DNQX for the MSR in the presence of APV or Mg^{2+} were not significantly different from each other, though both were different from the IC_{50}s of DNQX for the MSR and PSR in Mg^{2+}-free superfusate alone ($P < 0.01$). The IC_{50}s of DNQX for the latter two reflexes were significantly different from each other ($P < 0.01$) as well.

To further understand the change in the potency of DNQX caused by NMDA antagonists, the effect of Mg^{2+} was examined on the MSR and PSR in the presence of 1 μM DNQX (a concentration selective for the PSR; see Fig. 37). As the [Mg^{2+}] increased to 1.3 mM (from a trace quantity estimated at < 6 μM), the degree of inhibition of the MSR by DNQX was significantly ($P < 0.001$) enhanced (Fig. 40). For example, at 1 μM DNQX the MSR decreased 13% in a Mg^{2+}-free medium, but by 77% in 1.3 mM Mg^{2+} (Fig. 40). Similarly, the depression of the PSR by DNQX was greater as the [Mg^{2+}] was increased to 0.3 mM, the highest concentration at which a PSR could be routinely and reliably measured. The dose-response curves for Mg^{2+} on the MSR and PSR in the presence of 1 μM DNQX were not significantly different from each other (Fig. 40).

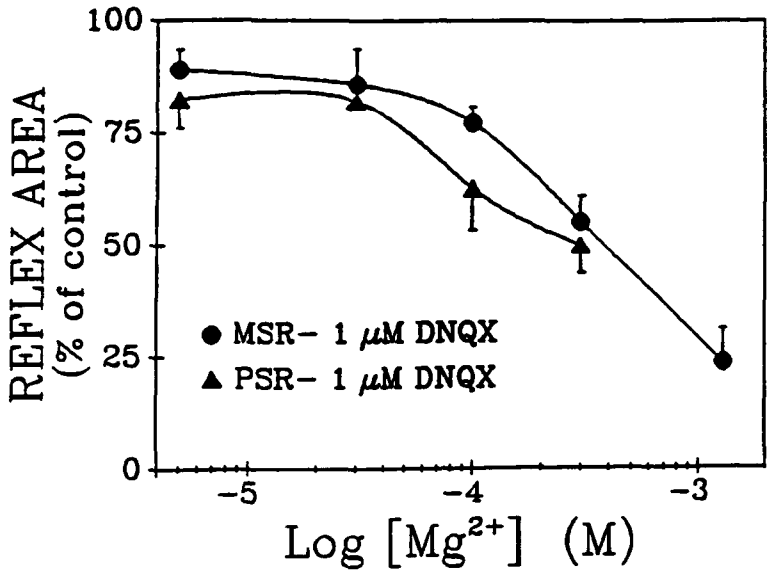

Fig. 40. Influence of DNQX on the dose-response relationship for Mg^{2+}-induced inhibition of the mono- and polysynaptic reflexes. Each point is the mean ± S.E.M. of 3-8 observations. Each observation at a given concentration of Mg^{2+}, was derived from a separate cord; data for up to 3 different concentrations may have come from a single cord using the semicumulative technique described in the methods.

In addition to affecting the magnitude of the reflexes, DNQX also affected their latencies. Unlike reflex area, the measurements of reflex latency were more difficult to accurately measure than was reflex area because it was more susceptible to slight variations in temperature[39] and reflex waveform. Nonetheless, DNQX increased the latency of both reflexes in the presence or absence of Mg^{2+} or when APV (10 μM) was present (Figs. 37,39 and 41). The increase in latency occurred at the same concentrations of DNQX that also depressed reflex activity. Neither APV (10 μM) nor Mg (1.3 mM) affected reflex latency (Figs. 37 and 39), and they did not alter the potency of DNQX-mediated increases in latency (Fig. 41) ($P > 0.05$).

The latency of the PSR was increased by the same amount of time as that of the MSR, except at the largest concentration of DNQX (6 μM) measured. In other words, the percentage increase in MSR latency was twice that of the PSR, even though the MSR was half the size of the PSR. Alternatively, this was expressed in the finding that the time interval between the MSR and the PSR was not affected by DNQX, except at the largest concentration of DNQX (6 μM) where the increase was 13 ± 5%. In contrast, the same concentration of DNQX increased the latency of the MSR by 53 ± 5%.

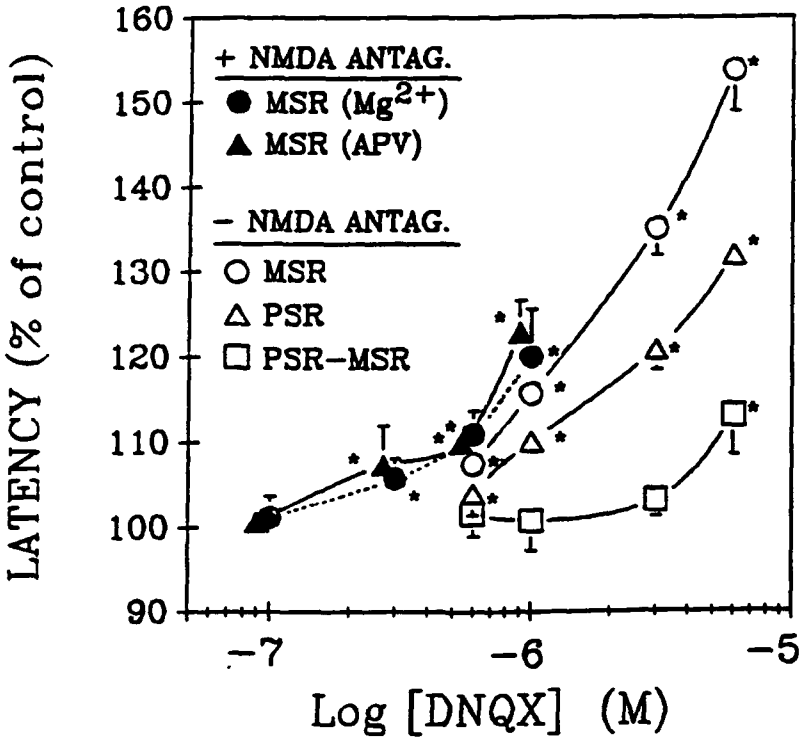

Fig. 41. Effect of NMDA antagonism on the dose-response relationship for alterations of reflex latency by DNQX. The values are given as the interval between stimulation and the first detectable point in the upstroke of the reflex (latency). MSR-PSR is the interval between the first detectable point in the upstroke of the MSR and PSR. The concentration of APV was 10 μM, while that of Mg^{2+} was 1.3 mM. Bars represent S.E.M. (N = 3-8). Data taken from records used in Figs. 37 and 39. The asterisks indicate $P < 0.05$ for DNQX treatment versus control (paired Student's *t*-test).

H. Alteration of Mono- and Polysynaptic Transmission by AChE Inhibitors

1. *Effect of DFP on Spinal Reflexes*

In the presence of Mg^{2+}, DFP (10-100 μM) significantly inhibited the MSR by 30-70%.[29] For example, the MSR was reduced by nearly 50% at 30 μM DFP (Fig. 42) in agreement with results previously obtained in this laboratory.[29] In the absence of Mg^{2+}, 6 μM DFP produced a small (5%) but statistically significant ($P = 0.008$) depression of MSR (Fig. 43). Increasing the concentration to 1 mM produced a maximal 9% depression of the MSR ($P = 0.011$). Atropine (200 nM) (n = 3) restored the MSR in cords treated with 100 μM DFP from 30% to 103% of control ($P < 0.05$ when compared to control) (Figs. 42 and 43).

In the absence of Mg^{2+}, DFP (3 μM -1 mM) depressed the PSR ($P < 0.05$) (Figs. 42 and 43). The depressant effect was maximal at 10 μM, where the reflex area was 36% of control and the MSR was significantly, but only mildly, depressed (50% depression of the PSR occurred at about 5 μM). Increasing the concentration of DFP to 1 mM resulted, paradoxically, in a partial

restoration of the PSR to 74% of control. The trend of reversal of depression was statistically significant for concentrations of DFP ≥ 60 μM (*i.e.*, the reflex area at 10 μM was significantly smaller [$P < 0.05$] than that at or > 60 μM). Atropine (200 nM) reversed the depression of the PSR in cords treated with 100 μM DFP from 68% to 112% of control ($P < 0.01$ with respect to DFP effect; $P > 0.05$ with respect to control).

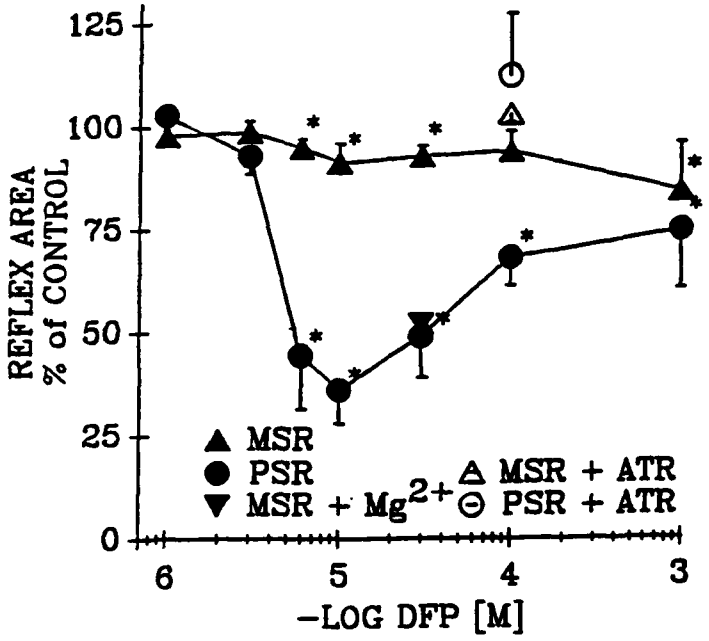

Fig. 42. Dose-response relationship for the effect of DFP on the mono- and polysynaptic reflexes. Mg^{2+} was absent for all experiments except for one set of experiments at 30 μM DFP when Mg^{2+} (1.3 mM) was added to determine the effect on MSR depression by DFP. Atropine (ATR) was added at 200 nM to cords treated with DFP (100 μM). The points are means ± S.E.M. of 3-6 observations; data obtained similarly to Fig. 40.

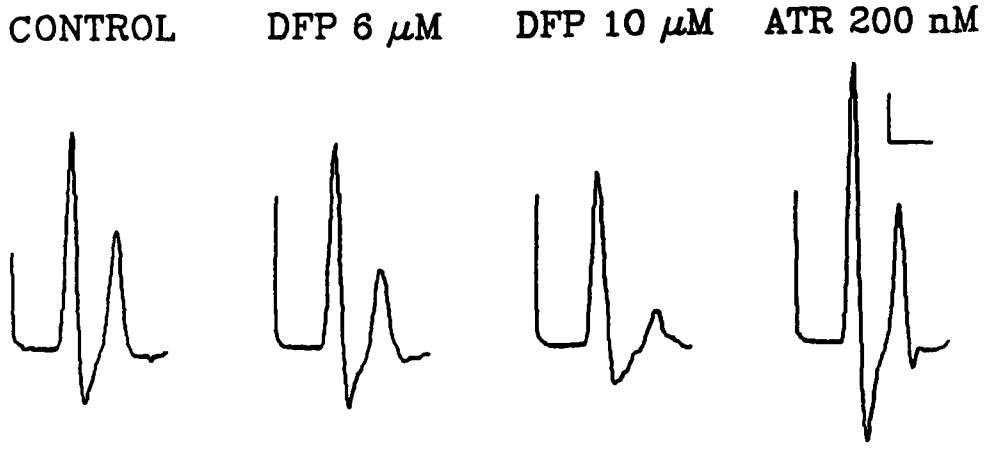

Fig. 43. Effect of DFP on the mono- and polysynaptic reflexes. The responses shown are signal-averaged traces of 5 successive reflexes in the absence of Mg^{2+} before, 30 min after exposure to DFP at each concentration, and 30 min after exposure to atropine. The first and second peaks after the stimulation artifact are, respectively, the MSR and PSR. Calibration: 1 mV and 5 ms.

2. *Effect of Physostigmine on Mono- and Polysynaptic Reflexes*

When Mg^{2+} was absent from the physiological solution, physostigmine (0.1 to 10 μM) had no effect on the MSR at concentrations that have been previously shown to depress the MSR by 90% when Mg^{2+} was present. Physostigmine (0.3 to 10 μM) significantly depressed the PSR ($p < 0.05$) in the absence of Mg^{2+} (Fig. 44). Maximum depression occurred at 10 μM physostigmine, which resulted in a depression of the PSR area to 46% of control. Increasing the concentration of physostigmine to 10 μM did not result in further depression. If anything, there was a slight trend of reversal similar to that seen with DFP, though this trend was not statistically significant (*i.e.*, values $\geq$ 0.6 μM did not differ significantly from each other).

3. *Effect of Sarin on the Mono- and Polysynaptic Reflexes*

In the absence of Mg^{2+}, sarin (10 nM to 1 μM) produced dose-dependent depression of both the MSR and PSR. The maximal depression of the MSR was to 21% of control while the PSR was completely blocked. Both reflexes were partially restored by subsequent exposure to atropine (200 μM); the MSR recovered to 75% of control and the PSR to 29% of control.

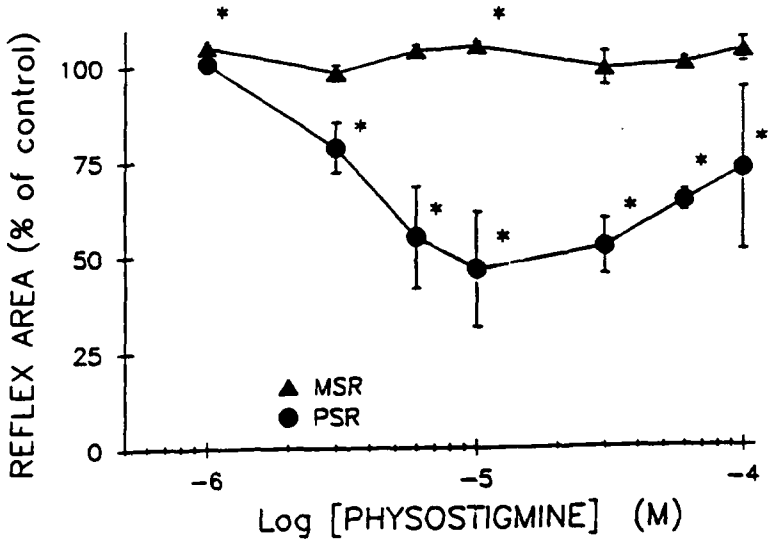

Fig. 44. Dose-response relationship for the effect of physostigmine on the mono- and polysynaptic reflexes in the absence of Mg^{2+}. Each point is the mean ± S.E.M. of 3-4 observations; asterisks indicate $P < 0.05$ with respect to control. Data were obtained similarly to Fig. 40. Values at concentrations less than 1 μM are not shown but had no effect on the reflexes.

VI. DISCUSSION AND CONCLUSIONS

A. DFP: Site and Mechanism of Depression in the Spinal Cord

The present study has examined the effects of DFP *in vitro* and sought to determine the role of AChE and of cholinergic receptors in the actions of DFP. DFP caused a concentration-dependent depression of the MSR and of total spinal cord ChE, but the MSR continued to decline in a dose-dependent manner after complete inhibition of ChE. Even though prolonged washing (2 hr) restored the MSR to its control level, ChE activity remained unchanged (*i.e.*, inhibited). In support of a possible direct muscarinic action of DFP, both atropine and benactyzine quickly (< 10 min) reversed the depression of the MSR by DFP. A previous report had in fact shown that the combination of atropine + benactyzine was distinctly more effective in counteracting the lethal effects of nerve agents *in vivo*, than when either drug was used alone.[128] This beneficial effect could be attributed either to the protection of both peripheral and central cholinergic receptors[128] or to a reduction of presynaptic stores of bound ACh.[138] Pretreatment with low nanomolar quantities of atropine not only reduced or prevented the segmental depression induced by DFP but even appeared to unmask a facilitatory action which we had not heretofore observed in the neonatal spinal cord. Such a facilitation of the MSR has been seen in cats given low doses (0.1-1.0 mg/kg, intrathecally) of DFP alone.[20] Sarin, an OP which is more potent than DFP in inhibiting ChE and in depressing the MSR, produces a facilitation of the MSR at low concentrations (2-20 nM) and depression at higher concentrations.[145,146] That depression is not only reversed or prevented by atropine but one can observe a concurrent appearance of facilitation in the presence of a normally depressant concentration of sarin. The mechanism of facilitation by sarin has not yet been adequately examined but it does not appear to involve cholinergic receptors (unpublished observations).

While muscarinic receptors are apparently involved in the spinal depressant action of DFP and other OP compounds (unpublished observations;)[145] the possibility exists that nicotinic receptors might also play a role in that depression. But we do not expect mecamylamine, a nicotinic antagonist, to reverse the depression of the MSR caused by DFP, since (+)-tubocurarine[145] or mecamylamine (unpublished observations, Yang and Warnick) did not prevent or reverse the depression of the MSR by sarin. The latter observations contradict the finding that mecamylamine can reverse PHY-induced depression of the MSR in cats.[55] But, conflicting reports exist on the efficacy of mecamylamine pretreatment in OP toxicity. While pretreatment with mecamylamine together with PYR and atropine offered protection against soman intoxication in rabbits,[70,73,95] it has also been reported that mecamylamine was ineffective in protecting rats from paraoxon toxicity.[41] Whether the beneficial effect of mecamylamine in OP toxicity *in vivo* or in its ability to reverse PHY-induced spinal depression is attributable to the protection of ACh recognition sites of central and/or peripheral nicotinic receptors, allosteric sites or associated ionic channels is uncertain. Clearly, however, the site of action of DFP on peripheral nicotinic receptors is not the recognition site for ACh. That site has been identified as an allosteric site on the nicotinic receptor of *Torpedo* electric organ[51] which in frog skeletal muscle appears to be located within the ionic channel of the nicotinic receptor.[91]

The results from this study do, however, confirm recent findings with DFP in the rat superior colliculus regarding the muscarinic nature of central OP actions. The increase in spontaneous activity in the rat superior colliculus by DFP could be blocked by pretreatment with atropine or

scopolamine whereas mecamylamine was ineffective,[19,86] leaving one to conclude that muscarinic receptors mediated the effect of DFP. Our earlier studies on the action of other OP compounds and cholinergic agonists on synaptic transmission in the spinal cord also support this interpretation. The dose-dependent depression of the MSR by tabun was also unrelated to inhibition of AChE and was similar to that caused by the cholinergic agonists carbamylcholine and oxotremorine.[131]

One question which arose from these studies regards the site and mechanism of OP-induced depression of segmental transmission. Three possibilities which have been proposed include: 1) the activation of Renshaw cells; 2) a direct effect on the motoneuron cell body; and/or 3) a presynaptic component. Previous studies which have examined the mechanism of depression by AChE inhibitors have discounted the role of Renshaw cells for two reasons: 1) motoneuron excitability increased during segmental depression rather than decreased and 2) antidromic field potentials increased during the depression of transmission.[55] A direct effect on the motoneuron soma would also seem unlikely because we have never observed an effect of various AChE inhibitors on the ventral root potential although small changes in potassium concentration invariably depolarize the ventral root.[38] Although remote, the possibility does exist that depolarization far from the cell body might go undetected and still contribute to segmental transmission. The possibility that a presynaptic mechanism is responsible for the depression of the MSR is supported by observations that cholinergic agonists alter the excitability of presynaptic terminals in the lateral geniculate nucleus[106] and that PHY altered the excitability of central terminals of Ia afferents leading to depression of the MSR in cat spinal cords.

In summary, DFP caused a dose-dependent depression of the MSR in spinal cords of neonatal rats which could be antagonized by muscarinic antagonists. Although it is doubtful whether nicotinic receptors play a role in the spinal action of DFP, the depression which DFP caused was unrelated to inhibition of AChE and appears to result from activation of muscarinic receptors located on or near presynaptic terminals.

B. Sarin: Mechanism of Facilitation and Depression of the Monosynaptic Reflex

It was previously thought that the acute neurotoxicity induced by OP agents such as sarin, soman, tabun and VX resulted solely from the 'irreversible' phosphorylation or phosphonylation of the esteratic site of AChE.[133] It was assumed that the accumulation of ACh, which followed inhibition of AChE by the OP agent, led to activation and then depression of central and peripheral cholinergic sites (*e.g.*, nicotinic synapses at autonomic ganglia and skeletal muscle and muscarinic receptors at parasympathetic end organs) and central cholinergic sites (*e.g.*, respiratory centers which are stimulated at low doses of OP but depressed at higher doses).[133] It has, in fact, been stated that "The stimulant and depressant effects [sic, of OP agents] are antagonized by atropine, although not as completely as are the muscarinic effects at peripheral autonomic effector sites."[133] But, the ability of atropine to block or reverse the adverse effects caused by the OP agents was considered an indirect action unrelated to the interaction between the OP agent and its substrate AChE.

The results of these studies on the spinal cord suggest that sarin in particular, and the OP agents in general, have at least two actions in the spinal cord relating independently to excitation (at

low concentrations) and depression (at higher concentrations). The depression of spinal monosynaptic transmission by sarin appears to be the result of a cholinergic action which is attributable to the activation of muscarinic receptors and not to the inhibition of AChE. The facilitation of the MSR by selected OP agents appears to result from a noncholinergic action (perhaps involving synaptic transmission at a strychnine-sensitive synapse or blockade of excitatory transmitter release from Ia afferents). The ability of muscarinic, but not nicotinic, antagonists to reverse or prevent OP-induced depression of monosynaptic transmission is indicative of the role of muscarinic receptors in modulating monosynaptic transmission, without having to attribute excitatory transmission to ACh itself. A number of possibilities arise, which although unproven at present, might explain the observed depressant effects. Among these are a presynaptic blockade of transmitter release from Ia afferents or a postsynaptic blockade of receptors (presumably glutamatergic).

The action of sarin, as well as DFP, might be attributed to accumulation of ACh subsequent to inhibition of AChE or to a direct effect of these agents on muscarinic receptors. Perhaps the most critical observations supporting a direct (non-AChE) action of muscarinic receptors are: 1) the similarity or response to sarin in normal and DFP-treated cords, despite the persistent inhibition of AChE; and 2) the apparent inability of pralidoxime to regenerate AChE while reversing the depressant effect of sarin on the MSR (*vide infra*).

The decrease in the MSR during repetitive stimulation in adult animal preparations *in vivo*[10] has been attributed to a failure of presynaptic function.[98] [The degree of depression was also greater when extracellular Ca^{2+} was decreased (data not shown).] These observations suggest that the phenomenon of homosynaptic depression that we observed *in vitro* shares a similar mechanism with that observed previously *in vivo*. These observations suggest that sarin can alter the amount of transmitter released per impulse at high frequency stimulation but a mechanism for that action has not yet been determined.

Thus we conclude that sarin depresses the MSR via muscarinic receptors unrelated to inhibition of AChE. Although M_1 and M_2 receptor antagonists are effective in reversing the depression caused by sarin, M_2 blockade appears to be most effective. Clonidine, an α_2-agonist which has been effective *in vivo* was ineffective in the cord suggesting that α_2-receptors in the cord do not play a role in the observed effects of the OPs. The receptors are probably located on the terminals of Ia afferents where they alter the release of glutamate. The excitatory effects of sarin appear to be unrelated to muscarinic receptors or cholinergic transmission but may be related to blockade of inhibitory transmission (*vide infra*).

C. Physostigmine and Pyridostigmine: "Reversible" Inhibitors of Cholinesterase

The reversible AChE inhibitors, PHY and PYR, each produced a concentration-dependent depression of the MSR which could be reversed or antagonized by atropine, which by itself was without effect.[55,123-125,146] The apparent activation of muscarinic, rather than nicotinic, receptors by the carbamates may have at least two possible alternatives: 1) a direct effect of PHY and PYR on a muscarinic receptor and 2) an inhibition of AChE yielding a local accumulation of ACh. In similar studies with the irreversible OP inhibitors, muscarinic antagonists but not nicotinic antagonists were effective in reversing or preventing segmental depression.[32,131,144-146]

It is important to note that the depressant effect of sarin on the MSR in the isolated spinal cord[146] and of DFP on the patellar reflex in cats[119] persisted against a background of ChE inhibition in the spinal cord. In addition, we have recently shown that pralidoxime, which purportedly acts through regeneration of AChE, reversed OP-induced segmental depression by sarin but failed to regenerate spinal cord ChE previously inhibited by sarin. Oximes, however, are also known to act as cholinergic antagonists in peripheral nerve and muscle.[2,3,87,93] It can be inferred from these results and previous work[32,123-125] that inhibition of ChE is not involved in the depressant action of the ChE inhibitors in the spinal cord and that the accumulation of ACh at synapses could not have been the cause of segmental depression with the carbamates. The observations suggest that the carbamates depress the MSR by an agonist action at muscarinic receptors.

With the exception of the nicotinic receptors on Renshaw cells, which have been well characterized in adult spinal cord,[25] the majority of cholinergic receptors in the central nervous system are of the muscarinic type and may exist pre- or postsynaptically. In the brain, these presynaptic muscarinic receptors are thought to function as autoreceptors regulating ACh release from nerve terminals.[67] Cholinergic receptors which are sensitive to atropine are also found on or near motoneurons in cats[149] and in the spinal cord of neonatal rats where both nicotinic and muscarinic receptors have been identified.[56] Whatever the role of these cholinergic receptors, they do not appear to directly function in primary segmental transmission, and the evidence for a pre- versus a postsynaptic site is not unequivocal.[120,149] Thus far, we have not been able to detect ventral root depolarization with the carbamates, sarin, or DFP at concentrations which were above the maximal blocking concentration (S. Das Gupta and J.E. Warnick, unpublished observations). Although this would seem to rule out a direct depolarization of the motoneuron soma, the possibility exists that the muscarinic receptors for the OPs and carbamates are located on the terminal dendrites of the motoneuron. If this were the case, we would not be able to detect small shifts in membrane potential. Alternatively, the OP- and carbamate-sensitive muscarinic receptors may exist on presynaptic terminals in close apposition to the motoneuron. In this manner the agonist action of the ChE inhibitors would be to release a substance which depresses motoneuron function. In support of such a possibility, the activation of muscarinic autoreceptors on dopaminergic nerve terminals in various brain regions leads to the release of dopamine[94,102] whereas activation of similar receptors on cholinergic nerve terminals leads to the inhibition of ACh release.[103,111] The activation of spinal dopamine receptors in cats and neonatal rats results in the depression of segmental transmission probably through α-adrenergic receptors.[17,88] Interestingly enough, DFP increases the turnover of dopamine in the rat striatum which was interpreted as being due to a secondary rise in ACh.[58]

Thus, it would appear that the depression of the MSR by the carbamates is unrelated to inhibition of ChE but results from a direct agonist action of the carbamates at muscarinic receptors. The location of these receptors is not yet determined but they may be situated on presynaptic terminals close to motoneurons where they release a substance which depresses segmental transmission or on dendrites of motoneurons where a direct depolarization would effectively cause a reduction in the MSR. Whether the toxicity of the OPs can be reduced or eliminated by pretreatment with the carbamates is also of interest and will be the subject of additional experiments.

D. Reversal of OP-Induced Reflex Depression by Thyrotropin-Releasing Hormone

Thyrotropin-releasing hormone (TRH; protirelin; p-Glu-His-Pro amide) is a hypothalamic factor which releases thyrotropin from the anterior pituitary and may also function as a neurotransmitter or neuromodulator in the central nervous system. Evidence for such a role includes the release of TRH from synaptosomes[9] and alteration of neuronal firing rate when TRH is applied iontophoretically.[47,118,142] Furthermore, radioimmunohistochemical studies have shown that TRH is widely distributed in the extrahypothalamic brain tissues of mammals[76,143] including the spinal cord where it is localized in both the dorsal and ventral roots.[101,126] Its localization in the spinal cord coupled with pathological findings of decreased TRH receptor binding in patients with amyotrophic lateral sclerosis have encouraged clinical investigations into its utility in the treatment of this disease.[15,53,54,71] In addition to its potential use as a therapeutic agent in neurologic disease, TRH has been shown to possess analeptic activity and is capable of reversing the effects of central depressants such as pentobarbital, phenobarbital and ethanol.[14,82,113]

Reports from this laboratory have revealed that TRH potentiated the MSR of spinal cords from neonatal male, but not female, rats in a concentration-dependent manner.[37] Our studies have also shown that several OP compounds including DFP and sarin cause a concentration-dependent depression of the MSR.[32,144-146] We were therefore interested in determining whether TRH could reverse OP-induced depression of the MSR. In this report we present evidence that TRH reverses DFP and sarin-induced depression of the MSR in spinal cords from male rats. The reversal occurs through an apparent noncholinergic, TRH-sensitive receptor mechanism and is unrelated to AChE activity. This action represents a potential use for TRH in OP toxicity.

This study confirms previous observations that DFP and sarin depressed the MSR in neonatal rat spinal cord, with 50% depression occurring at about 100 μM and 100 nM, respectively.[32,144] The depression caused by these concentrations of DFP and sarin were reversed by TRH. Maximal reversal of DFP's depressant effect occurred at 100 nM TRH while maximal reversal of sarin-induced depression required a 10-fold higher concentration of TRH (1 μM). The relative difference between TRH antagonism of DFP and sarin is probably related to the higher toxicity of sarin *vs.* DFP. Furthermore, high concentrations of TRH, when given in combination with the OPs, did not provide a greater degree of reversal. This reduced effect of TRH may be due to desensitization of TRH "receptors," an effect which has been reported earlier on rat spinal motoneurons, *in vivo*.[139] We also observed that atropine had no effect on the potentiation of the MSR caused by TRH although atropine does reverse both DFP- and sarin-induced depression of the MSR.[32,144]

The irreversible inhibition of AChE by OP compounds is known to occur through phosphorylation or phosphonylation of the serine hydroxyl at the active site of the enzyme. The resultant accumulation of ACh at both peripheral and central sites produces a variety of cholinergically related symptoms. Yet, anti-ChE compounds have been found to alter synaptic transmission by means other than inhibition of AChE. DFP, for example, directly blocks the ion channels of the nicotinic ACh receptor.[91,92] In both autonomic ganglia and neonatal rat spinal cord, the magnitude and time course of OP-induced alteration of synaptic transmission was not affected by prior 'irreversible' inhibition of AChE by DFP.[144,148] In these experiments, the concentrations of anti-AChE compounds that were used exceeded those necessary for inhibition

of AChE activity. Earlier work carried out in our laboratory has revealed that the muscarinic agonists oxotremorine and carbamylcholine act in a manner similar to the OPs causing depression of the MSR which is antagonized by atropine.[145] Thus, the ability of muscarinic antagonists (*e.g.*, atropine and pirenzepine) but not nicotinic antagonists (*e.g.*, D-tubocurarine and mecamylamine) to protect against or reverse OP-induced depression in the spinal cord indicates that muscarinic receptors have a major role in the acute neurotoxic actions of these compounds in the spinal cord.[32,131,145]

Several studies have implicated central cholinergic mechanism in the modulation of TRH interactions with central nervous system depressants. It is, however, debatable whether cholinergic receptors are involved in the ability of TRH to reverse either barbiturate-induced sedation or, for that matter, OP-induced depression in the spinal cord. The modulation of cholinergic transmission by TRH derives from studies in mice and rabbits in which antimuscarinic agents blocked arousal from pentobarbital-induced sedation by TRH.[14,84] Subsequently, Yarbrough[147] showed that TRH potentiated the postsynaptic effects of microiontophoretically applied ACh on cortical neurons. Conflicting results have been reported in the rat. In one study, pretreatment with atropine methyl bromide (intracerebroventricularly) completely blocked the antagonistic effect of intravenously administered TRH in pentobarbital-induced sleep time.[109] In another study, however, both atropine and scopolamine failed to block the reduction in pentobarbital-induced sleep time produced by TRH and its analog MK-771 (intracerebroventricularly) in the rat.[121] The results in the latter study were attributed either to a reduced sensitivity of cholinergic receptors to muscarinic blockade or a different neurochemical mechanism for TRH in the rat. In addition, the antipentobarbital action of TRH may also occur through a noncholinergic neural mechanism outside the hypothalamus.[107] In the present study, atropine did not affect TRH-induced potentiation of the MSR suggesting that in the rat spinal cord, there is no cholinergic involvement in the reversal of the analeptic effect of TRH.

The possibility that the extrahypothalamic actions of TRH in the nervous system occur through sites unrelated to the pituitary-type TRH receptor has been raised by a number of investigators. In examining the affinities of TRH and two of its analogues [DN-1417 and cyclo (HisPro)] for the TRH receptor in rat spinal cord and the brain, it was reported[71] that the acute transmitter-like action of these peptides was at non-TRH receptors since these compounds did not bind with high affinity and yet had strong physiologic and pharmacologic activities. The possibility that TRH was acting at a site unrelated to the pituitary-type TRH receptors was also suggested by Takahashi,[132] who found that TRH reduced voltage-dependent (delayed) K^+ conductance in rat spinal motoneurons via an action at potassium channels. Recent work from this laboratory has shown that two potent analogs of TRH (MK-771 and ^{3}methyl-His^2-TRH) were no different than TRH in potentiating the MSR in neonatal rat spinal cords. Cyclo (HisPro) did not have any potentiating effect on MSR (up to 100 μM) and TRH-free acid (pGlu-His-Pro) was 1000-fold less potent than TRH (Deshpande and Warnick, unpublished observations).

The results support the possibility that the reversal of OP-induced depression by TRH occurs through an apparent noncholinergic mechanism and is unrelated to AChE activity. These results also suggest that TRH and atropine act at dissimilar sites in the spinal cord, which further confirms the earlier work of Santori, *et al.*[121] Although controversial, TRH and its analogs are presently being used experimentally to treat patients with amyotrophic lateral sclerosis and other lower motor neuron disorders.[15,53,54,108] The findings from the present study suggest the possible

utility of TRH as an adjunct in OP intoxication and as a chemical tool in defining the mechanism of the central actions of OPs.

E. Mechanism of Oxime Reversal of Sarin-Induced Reflex Depression

The efficacy of quaternary bispyridinium oximes in treating OP toxicity has been attributed to their ability to reactivate peripheral AChE.[60,74,75,141] These compounds were therefore suggested as adjuncts along with a cholinolytic drug in counteracting OP toxicity.[74,75] However, the ability of the quaternary oximes to reactivate brain AChE as a mechanism for antidotal action in OP intoxication is controversial. On the one hand, quaternary reactivators such as pralidoxime exhibit low lipophilicity and do not easily penetrate the blood-brain barrier, although one study has shown results to the contrary.[59] But even pro-PAM, the lipophilic precursor of pralidoxime, provided only marginally greater survival against DFP than did pralidoxime and provided no protection against sarin[21] or paraoxon in mice,[72] even though it regenerated brain AChE.[72,127] Unlike most oximes, diethyxime is a tertiary reactivator of AChE and a reportedly universal antidote against OP intoxication at both central and peripheral sites.[90] The availability of a lipophilic oxime has therefore aroused some interest in one's ability to antagonize the central effects of OP compounds. That the antidotal action of the oximes might be attributed to a direct antagonism of ACh at nicotinic receptors as well as to reactivation of phosphorylated AChE was suggested by the structural similarity between the oximes and nicotine, and the nicotinic conformation of ACh suggests[129] and findings confirm that oximes possessed antagonist activities at both nicotinic and muscarinic receptors.[3,87,130] In fact, both pralidoxime and HI-6, another AChE reactivator, can modify the functional properties of the ion channel of the nicotinic ACh receptor.[2]

Both tertiary and quaternary oximes reversed the depression of spinal segmental transmission by sarin without causing a regeneration of spinal AChE. The order of potency of the three oximes in reversing sarin-induced inhibition in this study was: trimedoxime $\geq$ pralidoxime $>$ diethyxime. A comparison of the efficacy of pralidoxime, trimedoxime, obidoxime, and diethyxime revealed that the latter agent was ineffective in acute intoxication by DFP,[31] but effective against the peripheral effects of weaker inhibitors of AChE.[45] In the present experiments, however, diethyxime exhibited significant reversal of the segmental depression caused by sarin which approached that of the other oximes, while none of the oximes examined effectively regenerated AChE.

Although the therapeutic efficacy of oximes has been commonly related to their ability to dephosphorylate inactivated AChE,[74] the possibility exists that they may act on the ACh receptor. The structural similarities of oximes with ACh,[129] their nicotinic and muscarinic antagonist activities at central and peripheral sites[3,87,89,93], and their ability to block the nicotinic ion channel in frog skeletal muscle[2] all suggest that the oximes are cholinergic antagonists. Furthermore, pralidoxime can both facilitate and depress transmission in rat diaphragm and frog sartorius muscle, suggesting a dual action at the neuromuscular junction through its weak anti-ChE and channel-blocking action.[2,46]

Our previous studies demonstrated that depression of the MSR in neonatal rat spinal cord by sarin, soman, and DFP could be reversed by muscarinic, but not by nicotinic, antagonists.[145,146]

Furthermore, the magnitude and time course of OP-induced alterations of synaptic transmission in both isolated autonomic ganglia and spinal cords persisted against a background of AChE inhibition.[145,146,148] That regeneration of AChE is therefore not a prerequisite to the reversal of OP-induced central depression was also apparent in the ability of TRH to reverse DFP-induced depression.[29]

It is evident from this study that reactivation of AChE is not a factor in reversing the sarin-induced depression of MSR in neonatal rat spinal cord. Instead, the reversal of OP-induced depression by oximes most likely results from an antimuscarinic action.

F. Role of Inhibition in Organophosphorus-Induced Facilitation

Upon examining the action of several OP and carbamate inhibitors of AChE on the MSR in neonatal rats, *in vitro*, it was noted that sarin facilitated the MSR by 50% at low concentrations (2-20 nM), while it depressed the reflex at higher concentrations.[144-146] In similar studies with tabun, the degree of facilitation was much reduced in comparison with sarin,[131] while soman, DFP, PHY, and PYR did not cause potentiation.[27-29, 144] In addition, both oxotremorine and carbamylcholine were previously shown to cause a facilitation and depression similar in magnitude to tabun, but at much higher concentrations.[131]

The depression of the MSR by these AChE inhibitors and muscarinic agonists could be either reversed or prevented by muscarinic antagonists but not by nicotinic antagonists. Furthermore, the depression of the reflex by the OP compounds persisted against a background of AChE inhibition.[32,146] While the depression by sarin could also be reversed by bis-pyridinium oximes, AChE was not regenerated simultaneously.[27] Of particular interest was our observation that the OPs and carbamates could also cause a paradoxical facilitation of the reflex at depressant concentrations in the presence of atropine, which by itself had no effect.[28,29,137,144,145] These observations led us to propose that the OP and carbamate inhibitors of AChE affect segmental transmission by activation of a muscarinic receptor most likely coupled to dopamine release,[27,94,103,104] that protective carbamylation of ChE is ineffective against OP-induced segmental depression, and that inhibition of ChE is unrelated to both carbamate- and OP-induced depression of the MSR.

Although the probable site and mechanism of OP- and carbamate-induced depression of segmental transmission have been examined, the mechanism of facilitation has eluded us until now. Obvious possibilities include a direct effect on the motoneuron (*e.g.*, depolarization), the release of a putative excitatory transmitter (*e.g.*, glutamate), and blockade of inhibition. Recently, we described the existence of inhibition in the spinal cord of neonatal rats, which consisted of an early and a late phase of inhibition, which could be blocked by strychnine and bicuculline, respectively. The early phase of inhibition may be of postsynaptic origin and mediated by glycine, while the late component of inhibition may be presynaptic in origin and mediated by gamma-aminobutyric acid (GABA).[39]

In the hemisected spinal cord preparation, the MSR was inhibited by stimulation applied to an adjacent dorsal root. That inhibition reached a maximum when conditioning-test intervals were 3-5 msec, and inhibition was still apparent, yet significantly reduced, when these intervals were

of the order of 25-30 msec. The inhibition is apparently chloride-dependent and can be blocked by strychnine, which leads to the suggestion that it is of postsynaptic origin.[39,116] Pharmacological studies on this inhibitory pathway previously revealed the existence of two types of inhibition in the spinal cord of neonatal rats, which have usually been associated with either pre- or postsynaptic inhibitory mechanisms.[39] In contrast to earlier reports on this process in neonatal cords, we found that this inhibition had two distinct phases: a fast-rising early phase and a more delayed, long-lasting late phase.[39] The early phase of inhibition, which is strychnine-sensitive, corresponded to the time course of postsynaptic inhibition in cats,[122] appeared to reflect the time course of inhibitory postsynaptic potentials,[48] could be evoked synaptically by activation of glycinergic interneurons,[23] and exhibited conductance changes which can be mimicked by microiontophoretically applied glycine. The inhibitory potentials are typically of short latency, having an onset of 1-2 msec, with peak effects at 5-6 msec, and lasting 20-25 msec.[48] In both cases, the inhibitory potentials and glycine-induced changes on α-motoneurons were blocked in a competitive manner by strychnine.[24,48] These results suggest that the neurotransmitter involved in the pathway for this early phase of inhibition in the neonatal cord is, most likely, glycine. The later phase of inhibition in the neonatal cord exhibits similarities in time course to that of presynaptic inhibition[49,50] and it too is blocked by the GABA antagonist bicuculline. In comparison with postsynaptic inhibition, presynaptic inhibition has a longer latency (2-4 msec), its peak effect occurs at 20-30 msec, and lasts for 150-200 msec.[49] Both bicuculline and picrotoxin block this type of inhibition by interacting with GABA receptors,[23,96] while semicarbazide reduces the magnitude of presynaptic inhibition by depleting the amount of presynaptic transmitter.[8] Thus, it would appear that this late inhibition in the neonatal cord is a presynaptic inhibition mediated by GABA-ergic neurons. A decrease in extracellular Cl^- blocks both pre- and postsynaptic inhibitions in mammalian spinal cord[6,33] and postsynaptic inhibition in the neonatal cord.[116] The time course of both glycine- and GABA-mediated changes of Cl^- ion conductance determined in cultured mouse spinal neurons using voltage clamp is similar to the time course that we have observed here, *i.e.*, an early phase of glycine-mediated Cl^- ion conductance with a half-decay time of 60 msec and late phase of conductance mediated by GABA with a half-decay time of 330 msec.[7] Therefore, it is interesting to note that both pre- and postsynaptic inhibition in the neonatal rat spinal cord, apparently mediated by glycine and GABA, respectively, can be mediated via a similar -- yet temporally dispersed -- pathway.

The reduction of the late (bicuculline-sensitive) phase of inhibition by low concentrations of sarin (3-20 nM) which facilitated the MSR, but had no effect on the early (strychnine-sensitive) phase of inhibition, is indicative of a presynaptic mechanism. At concentrations of sarin ($\geq$ 30 nM) which depressed the MSR, the late phase of inhibition was either blocked to a lesser extent or enhanced. Although the depression of the MSR has been attributed to activation of a muscarinic receptor and is unrelated to inhibition of AChE, the cause of the facilitation has heretofore remained unknown. It appears that the facilitation of the MSR by sarin may be caused by a blockade of a bicuculline-sensitive inhibition which is most likely mediated by GABA.

G. Role of Glutamatergic Receptors in Reflex Spinal Transmission

Relatively low concentrations ($<$ 2 mM) of Mg^{2+} are known to interrupt or mask the NMDA-mediated responses in central nervous system preparations *in vitro* (including the spinal

cord).[4,22,64,105,134,136] Thus, the removal of Mg^{2+} from the physiological solution bathing the isolated spinal cord allowed for the appearance of the PSR and an examination of the action of OPs on polysynaptic *vs.* monosynaptic transmission, which is purportedly mediated via *non*-NMDA (quisqualate and kainate) receptors.[4,18,112] The actions of NMDA-specific antagonists were also studied to assess the contribution of glutamate receptor subtypes and receptor-specificity in transmission through mono- and polysynaptic pathways. In this regard, DNQX was considerably more potent in depressing the MSR in the presence of NMDA antagonists (Mg^{2+}, APV, or dizocilpine) than in their absence and it was potent in depressing the PSR (in the absence of Mg^{2+}). Although the depression of both reflexes at low concentrations of DNQX was similar, there was a significantly greater depression of the PSR relative to the MSR at high concentrations of DNQX. In addition, the latency of both reflexes was similarly increased by DNQX, whereas Mg^{2+} and APV had no effect on MSR latency or on the DNQX-mediated increase in latency.

The AChE inhibitors action on reflex activity in the absence of Mg^{2+} diverged from that seen in the presence of Mg^{2+}. For example, while DFP and PHY did cause significant depression of the PSR (in the absence of Mg^{2+}), they had no effect on the MSR at concentrations which previously caused complete inhibition of the MSR in the presence of Mg^{2+}. Sarin likewise caused little depression of the MSR, while completely blocking the PSR. In any case, the ability of atropine to reverse both MSR and PSR depression suggests some cholinergic involvement.

The remaining discussion deals with two questions: (1) What is the role of NMDA and *non*-NMDA receptors in the mono- and polysynaptic pathways? (2) Can the site of action of AChE inhibitors in affecting reflex activity be identified? Although one can reach reasonable conclusions about the receptor types involved in reflex activity, insufficient data are available to attempt any conclusive statements about the depression of reflex activity by inhibitors of AChE.

1. NMDA Receptor Types

In the embryonic rat cord, monosynaptic activation of motoneurons evokes a fast-rising potential mediated by *non*-NMDA receptors superimposed upon a slower-rising potential mediated by NMDA receptors.[150] On the other hand, polysynaptic activation elicits only a slow-rising potential of long duration mediated by NMDA receptors.[150] Neurons exhibiting such composite potentials that are mediated by both NMDA and *non*-NMDA receptors are also present in other systems.[100] Blocking one component of a composite potential should reduce the probability of generating an action potential and cause a significant reduction in a population reflex such as the MSR or PSR. The similar degree of depression of the MSR by selective blockade of either NMDA or *non*-NMDA receptor is consistent with this notion. However, the simultaneous blockade of both *non*-NMDA and NMDA potentials would make it highly unlikely that the threshold for action potential generation could be reached. The combination of NMDA and *non*-NMDA receptor blockade should potentiate depression of reflex activity. Such an effect was demonstrated herein (*vide supra*). Similarly, a synergistic interaction between the *non*competitive NMDA antagonist ketamine and DNQX has also been reported in the cat spinal cord *in vivo*.[80] Thus, these findings are consistent with the activation of both NMDA and *non*-NMDA receptors of motoneurons upon monosynaptic excitation.

In the presence of NMDA blockade, the resulting MSR is believed to be mediated by *non*-NMDA receptors.[5,34,26,63,83,99,114,117] Therefore, the depression of MSR by DNQX in the presence of Mg^{2+}, dizocilpine, or APV represents blockade of *non*-NMDA receptors by DNQX. In fact, in the presence of these agents, the potency of DNQX in depressing the MSR is in agreement with values for DNQX blockade of quisqualate-mediated responses which occur in other spinal and brain preparations.[13,43,44,61,80,81] In addition, the monophasic nature of the dose-response curves suggests that a single receptor or a set of receptors with a single affinity was involved. This result is compatible with the previous observations on rat and cat spinal cords, respectively, in which the responses elicited by α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) or quisqualate and kainate were blocked by DNQX in an equipotent manner.[12,13,80,81] Since we did not observe a biphasic depression of the MSR by DNQX during NMDA blockade, our results appear to be consistent with the hypothesis that the electrophysiological responses of *non*-NMDA receptors are mediated via one receptor complex.[80,81] However, we cannot rule out the possibility that our results may reflect the predominance of a single *non*-NMDA receptor subtype in the MSR.

At higher concentrations, DNQX also blocks NMDA receptors.[12,13,43,44,61,79,80] In the experiments presented here, DNQX reduced the magnitude of the MSR by 50% at ≈ 4 μM in the absence of NMDA blockade. Coincidentally, a significant reduction of NMDA-induced depolarization in the spinal cords of neonatal rats occurred at 10 μM DNQX.[12,13] Thus, at this high concentration of DNQX, both NMDA and *non*-NMDA receptors were blocked. This reinforces the notion that a simultaneous block of both NMDA and *non*-NMDA receptors must occur before there is a sizable depression of the MSR. Therefore, the 15- to 20-fold change in potency of DNQX-mediated depression of the MSR in the presence and absence of NMDA blockade represents the index of selectivity of DNQX for NMDA *vs. non*-NMDA receptors. This index of selectivity was similar to those reported in the other systems above.

The difference in latency between the MSR (4.8 msec) and PSR (8.8 msec) represents, in part, the time taken for synaptic transmission across a one- and two-neuron arc, respectively. DNQX increased the latency of both reflexes concurrent with reflex depression but irrespective of the presence of NMDA blockade. Furthermore, Mg^{2+}, APV, and dizocilpine did not alter MSR latency or potentiate the increase in reflex latency caused by DNQX. These results were similar to the increase in latency of cochlear potentials in the guinea pig that occurs during blockade of *non*-NMDA receptors by DNQX and CNQX,[69,97] the increase in latency of kainate-evoked bursts in adult rat hippocampal slices by CNQX, and the failure of D-AP7 to affect latency.[110] Therefore, the increase in reflex latency appears to be correlated with blockade of *non*-NMDA receptors and to be independent of the presence or absence of NMDA receptor blockade.

The identity of the receptors mediating synaptic transmission between a dorsal root afferent neuron and an excitatory interneuron (the first synapse) is unknown, while NMDA receptors are known to mediate transmission between the interneuron and motoneuron (the second synapse).[57,150] Our results suggest that neurotransmission across this first synapse of the PSR occurs through both NMDA and *non*-NMDA receptors. Since low concentrations of DNQX (1 μM) are selective for *non*-NMDA receptors, the similarity in the magnitude of depression of the MSR and PSR must be due to blockade of *non*-NMDA receptors. Furthermore, DNQX increased the latency of the PSR by the same amount of time that it increased the MSR, and

DNQX-mediated increases in latency correlate with the presence of *non*-NMDA receptors. Because this second synapse is mediated solely by NMDA receptors, the *non*-NMDA receptors must be present only at the first synapse. It seems likely that both NMDA and *non*-NMDA receptors must be present at the same synapse for synergistic depression to occur from a combination of NMDA and *non*-NMDA antagonists. Such depression of the PSR by Mg^{2+} and DNQX suggests that, like the synapse in the monosynaptic pathway, both NMDA and *non*-NMDA receptors are present at the first synapse, while the second synapse of this arc is mediated solely by NMDA receptors. The greater depression of the PSR relative to the MSR at concentrations of DNQX that block both NMDA and *non*-NMDA receptors is consistent with such a model. With two synapses in series, blockade of transmission at either the first or the second synapse would reduce the resultant reflex. When both synapses are blocked, the transmission through the entire reflex arc will be synergistically depressed.

How much error is introduced by using Mg^{2+}-free superfusate to unmask the NMDA responses? Conceivably, errors could be associated with a reduction in action potential threshold and an increase in synaptic release of neurotransmitter in Mg^{2+}-free conditions.[150] Such alterations should augment reflex activity in Mg^{2+}-free conditions, and therefore lower the potency of depression of reflex activity by DNQX. Both Mg^{2+} and APV (10 μM) cause complete NMDA blockade so that any difference in the potency of depression of the MSR by DNQX could be attributed to this effect. In fact, there was a small but statistically insignificant ($P > 0.05$) difference in potency in the direction predicted (IC_{50}s: 1.3 mM Mg^{2+} = 0.70 μM *vs.* Mg^{2+}-free + APV = 0.93 μM). Thus, any bias introduced by Mg^{2+}-free conditions appears to be minimal.

Thus, these studies on reflex activity in the spinal cord of the neonatal rat indicate that both NMDA and *non*-NMDA receptors are present at the synapses of both the afferent neuron and the excitatory interneuron in the PSR, and the afferent neuron and the motoneuron in the MSR. DNQX is a 15- to 20-fold more potent antagonist of *non*-NMDA than of NMDA responses.

2. The Inhibitors of Acetylcholinesterase and Glutamatergic Transmission

The removal of Mg^{2+} from the physiological solution allows the generation and visualization of the polysynaptic component of the dorsal-ventral reflex. The removal of that divalent cation, however, renders the monosynaptic pathway unresponsive to DFP and PHY, but not to sarin. Whereas DFP and PHY caused 50% inhibition of the MSR (with Mg^{2+} present) at 80 μM and 0.45 μM, respectively, only minimal effects on the MSR were seen in the absence of Mg^{2+} at concentrations of DFP and PHY as high as 1 mM and 100 μM, respectively. These same concentrations substantially reduced the PSR. There appeared to be no difference in the dose-response curve for sarin (preliminary observations) whether or not Mg^{2+} was present.

There also appeared to be a moderate-sized component of the PSR (35-45%) which was resistant to inhibition by DFP (up to 1 mM) or PHY (10 μM); concentrations which were greater than those that caused maximum inhibition of the nonresistant component. Sarin, on the other hand, caused complete inhibition of the PSR and about 80% inhibition of the MSR in the absence of Mg^{2+} (similar to the inhibition of the MSR in the presence of Mg^{2+}). Thus, there appears to

be some significant differences between the carbamate and weak OP inhibitors of AChE and an agent such as sarin.

The fact that these compounds have their maximum effects on the MSR and PSR at concentrations far less than those that inhibit 90% of the AChE (in the presence of Mg^{2+}) suggests that these effects are not due to either inhibition of AChE or an excess of ACh, but are instead due to direct effects. The observation that atropine completely reverses the effects of DFP on the MSRs and PSRs and partially reverses the effects of sarin on the MSRs and PSRs indicates that muscarinic receptors are definitely involved (either directly or indirectly) in the depressant actions of the AChE inhibitors. With the limited data at hand, one can also infer that the carbamates and OPs preferentially act at NMDA rather than at *non*-NMDA sites. This is supported by the differential action of the AChE inhibitors on monosynaptic *vs.* polysynaptic reflexes. The exact interrelationship, if any, between muscarinic and NMDA receptors cannot be determined at this time and with the data at hand. Clearly, though, modulation of the NMDA receptor by Mg^{2+} significantly affects the ability of OPs and carbamates to depress monosynaptic transmission and may suggest that the various forms of glutamatergic receptors are similar and that one can modulate the predominance in any one pathway. The latter findings, together with the paradoxical observation that increasing the concentration of AChE inhibitor in the absence of Mg^{2+} eventually results in reversal of depression, are not easily explained at this time and must await further investigation.

VII. REFERENCES

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ACETYLCHOLINESTERASE INHIBITORS ON THE SPINAL CORD
Subtitle: Actions of Organophosphates in the Mammalian Spinal Cord

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PUBLICATIONS

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